NAMIBIA UNIVERSITY
OF SCIENCE AND TECHNOLOGY

PREVALENCE AND MOLECULAR CHARACTERISATION OF GROUP B STREPTOCOCCUS IN PREGNANT WOMEN FROM HOSPITALS IN OHANGWENA AND OSHIKOTO REGIONS OF NAMIBIA

By

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Thesis submitted in fulfilment of the requirements for the degree of Master of Health Sciences, Faculty of Health and Applied Sciences, Namibia University of Science and Technology, Windhoek, Namibia

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March 2019

DECLARATION

I, Erastus Lafimana Haimbodi hereby declare that the work contained in the thesis entitled Prevalence and molecular characterisation of Group B streptococcus in pregnant women from hospitals in Ohangwena and Oshikoto regions of Namibia is my own original work and that I have not previously in its entirety or in part submitted it at any university or other higher education institution for the award of a degree.

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DEDICATION

I GRATEFULLY DEDICATE THIS WORK TO:

My parents Mr and Mrs Haimbodi

Thank you for bringing me up to be where I am today. I adore you.

and

All mothers who participated in this study

Thank you for being there, this study could have been in vain without your participation.

ABSTRACT

BACKGROUND

Group B streptococcus (GBS) naturally colonises the lower gastrointestinal and female genitourinary tract. It poses a great risk of morbidity and mortality to infants born to colonised mothers. It is highly implicated in maternal infections such as endometritis and chorioamnionitis as well as neonatal infections such as pneumonia, meningitis and septicaemia. It also causes stillbirths and miscarriages in colonised women.

Group B streptococcus infection in neonates is classified in two clinical syndromes, which are Early Onset Disease (EOD) and Late Onset Disease (LOD). Early Onset Disease presents between day zero and day seven, and LOD between day eight and day ninetieth (third month) of the infant's life. GBS is classified into capsular types Ia, Ib and II – IX, based on the polysaccharide capsular antigens of the bacterium. This study aimed at determining the prevalence rate, antimicrobial susceptibility patterns and molecular characteristics of *Streptococcus agalactiae* isolated from pregnant women at 35 weeks of gestation and above, who attended antenatal screening at selected hospitals in Ohangwena and Oshikoto regions of Namibia.

METHODOLOGY

A descriptive cross-sectional study was conducted from May to September 2018 at selected hospitals. A convenient sampling technique was used to recruit 210 pregnant women at 35+ weeks of gestation. Descriptive data was collected using structured questionnaires. In total, 210 lower vaginal and rectal swabs were collected from participants by trained registered nurses, and were submitted to the laboratory at 2 – 8°C for culture. Swabs were cultured onto 5% sheep blood agar and into Todd-Hewitt broth, both with colistin and nalidixic acid.

Plates were examined for the growth of whitish-grey and translucent colonies with big or narrow zone of beta haemolysis. Probable colonies were identified as GBS using the Christie, Atkins, Munch and Petersen (CAMP) test and polymerase chain reaction (PCR). Antimicrobial sensitivities were determined using Kirby-Bauer disc diffusion method and Vitek 2 methods. Gene based resistance and capsular types were examined using PCR. Statistical analysis was performed using the Pearson's Chi square test, and a P value < 0.05 was considered statistically significant.

RESULTS

Twelve (5.7%) of women screened were colonised by GBS, of which 25.0% were colonised rectovaginally, 58.0% vaginally and 17.0% rectally. No significant association was reported between GBS colonisation and maternal age, habitat, marital status, education, employment, parity, still births and miscarriages (*P* values >0.05). Antimicrobial susceptibility was reported at 100% for ampicillin, penicillin & ceftriaxone. Resistance to tetracycline was reported at 100%. Tetracycline resistance gene *tet*(M) was present in 88.9% of the isolates only and none of the isolates presented with *tet*(O). Polysaccharide capsular type Ia was found in 9(50%) and Ib was found in 1(5.5%) of the total isolates. The remaining isolates were not typeable using PCR.

CONCLUSION

Streptococcus agalactiae's positive rate was 5.7% among the pregnant women examined. Socio-demographic and obstetric factors had no influence on GBS colonisation (*P* values >0.05). No resistance was reported to ampicillin, penicillin and ceftriaxone. No sensitivity was reported to tetracycline. Fifty (50%) of the isolates were capsular type Ia, 5.5% were type Ib and 44.4% were not typeable using PCR.

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µg	: Micro gram
µl	: Micro litre
µM	: Micro moles
ml	: Millilitre
mm	: Millimetre
Rpm	: Revolutions per minute
B	: Beta
µg/ml	: Microgram per millilitre
Bp	: Base pairs

LIST OF ABBREVIATIONS

ATCC	: American Type Culture Collection
BBB	: Blood Brain Barrier
BCNA	: Columbia Sheep Blood with Colistin and Nalidixic acid
BibA	: GBS immunogenic bacterial adhesin
CAMP	: Christie, Atkins, Munch and Petersen test
CDC	: Centres for Diseases Control and Prevention
CLSI	: Clinical Laboratory Standards Institute
DNA	: Deoxy Ribonucleic Acid
EOD	: Early Onset Disease
Fbs	: Fibrigogen binding proteins
GBS	: Group B *Streptococcus*
HIV	: Human Immuno-Deficiency Virus
IAP	: Intrapartum Antibiotic Prophylaxis
Lmb	: Laminin binding protein
LOD	: Late Onset Disease
LVS	: Lower Vaginal Swab
MIC	: Minimum Inhibitory Concentration
NIP	: Namibia Institute of Pathology
NUST	: Namibia University of Science and Technology
PCR	: Polymerase Chain Reaction

rRNA	: Ribosomal Ribonucleic Acid
S. agalactiae	: *Streptococcus agalactiae*
S. aureus	: *Staphylococcus aureus*
SBA	: Sheep Blood Agar
*Scp*B	: Group B streptococcal C5a peptidase
SfbA	: Streptococcal fibronectin binding protein
SPSS	: Statistical Package for Social Sciences
S. pyogenes	: *Streptococcus pyogenes*
TAE	: Tris-Acetate EDTA buffer
Taq	: Polymerase enzyme produced by *Thermophilus aquaticus*
TH	: Todd-Hewitt broth

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Streptococcus agalactiae (*S. agalactiae*) is a gram-positive bacterium which belongs to the Lancefield Group B streptococcus (GBS). It is part of the normal flora of the gastrointestinal and female genitourinary tracts, and it is present in the genital tract of about 20 – 60% of pregnant women (Alemseged *et al*., 2015). Nearly 20 – 40% of healthy women are colonised by GBS, and 50 – 70% of infants born to these mothers become infected with GBS (Doran & Nizet, 2004).

Group B streptococcus is a leading cause of neonatal sepsis and meningitis worldwide (Heelan *et al*., 2004). It is widely implicated in maternal infections such as endometritis and chorioamnionitis as well as neonatal infections such as pneumonia, meningitis, sepsis and septicaemia (Gygax *et al*., 2006). Mortality of GBS in neonates is over 50% and is particularly high in preterm infants (Heelan *et al*., 2004). Vaginal colonisation by GBS during pregnancy is associated with premature delivery and still births (Alemseged *et al*., 2015).

The median prevalence of GBS in pregnant women in Africa was reported at 16% in 2009 (Joachim *et al*., 2009). The prevalence in Windhoek, Namibia was reported at 13.6% in 2016 (Engelbrecht *et al*., 2016). Furthermore, the colonisation rate in other Southern African countries was reported at 31.6% in Zimbabwe in 2000 (Moyo *et al*., 2000), 30.9% in South Africa in 2012 (Bolukaoto *et al*., 2015; Monyama *et al*., 2016), 21.2% in Malawi in 2010 (Gray *et al*., 2011) and 1.8% in Mozambique in 2008 (de Steenwinkel *et al*., 2008).

Most studies done in Africa have shown a uniformity in sensitivity of GBS to penicillin and ampicillin, the recommended drugs of choice against GBS. The Centres for Diseases Control and Prevention (CDC) requires GBS screening in women at 35 – 37 weeks of gestation. Women colonised by GBS, who deliver infants with invasive GBS disease, those having GBS during gestation including those at risk but of unknown GBS status at delivery are given prophylaxis to prevent neonatal infection. Penicillin or ampicillin are the antibiotics of choice for prophylaxis and treatment of GBS infection. However, erythromycin & clindamycin are recommended for patients with a beta (β)-lactam allergy (CDC, 2010).

Group B streptococcus is classified into ten common capsular types based on their polysaccharide capsular antigens on the surface of the organism. The capsule is the main virulence factor for GBS. These capsular types are Ia, Ib, II, III, IV, V, VI, VII, VIII and IX (Wang *et al*., 2015; Teatero *et al*., 2014). The most prominent capsular types in Southern Africa are type Ia, Ib, II, III and V (Belard *et al*., 2015; Chukwu *et al*., 2015; Kwatra *et al*., 2014; Moyo *et al*., 2000; Mavenyengwa *et al*., 2010). Gene based resistance in GBS is encoded by *ermB, ermA, ermTR, mefA, mefE, aphA-3, aad-6, tet(M), tet(O), int-Tn* and *mreA* (Zeng *et al*., 2006). The prevalence and molecular characteristics of group B streptococcus isolates are elucidated in this study.

1.2 LITERATURE REVIEW

1.2.1 Characteristics and virulence factors of GBS

Streptococcus agalactiae is a Group B beta-haemolytic streptococcus which possesses a Lancefield Grouping B antigen, a type-specific cell-surface polysaccharide and protein antigen. The organism is covered with a sialic acid-rich anti-phagocytic polysaccharide capsule, which acts as the main pathogenic and virulence factor. In addition, it produces pore-forming toxins, which are crucial for its ability to cause invasive diseases (Winn *et al*., 2006). GBS is classified into ten common types based on the capsular polysaccharide antigens. These are Ia, Ib, II, III, IV, V, VI, VII, VIII, IX (Wang *et al*., 2015; Teatero et al., 2014).

GBS has several virulence factors which enable it to colonise and invade host cells to cause infection. The persistent colonisation and invasion of GBS into host cells widely depends on the presence of adhesin protein molecules on its cell surface. This adhesion proteins include the fibrinogen-binding proteins (Fbs), Laminin-binding protein (Lmb), group B streptococcal C5a peptidase (ScpB), streptococcal fibronectin-binding protein A (SfbA) and the GBS immunogenic bacterial adhesin (BibA) (Shabayek & Spellerberg, 2018). Moreover, the organism possesses surface-protruding structures like pili which are essential in promoting colonization and invasion of the central nervous system (Shabayek & Spellerberg, 2018). Figure 1.1 depicts the major adhesin proteins which mediate GBS interaction with host cells.

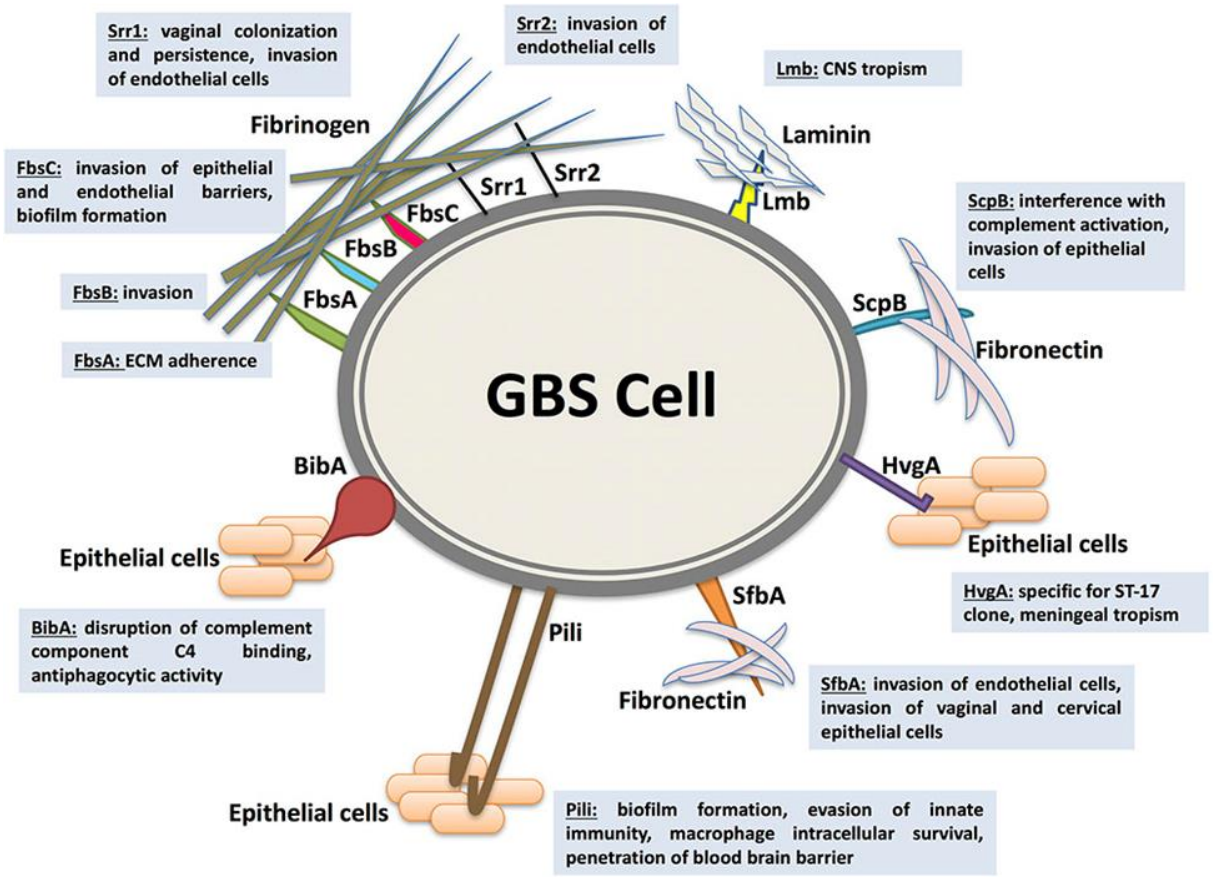

Figure 1.1: Major adhesin molecules that mediate GBS interaction with host cells
Adapted from Shabayek & Spellerberg, 2018

1.2.2 Pathogenesis of GBS infection

Group B streptococcus colonises the genital and gastrointestinal tracts of healthy women asymptomatically. Vaginal GBS colonisation is said to be secondary to the gastrointestinal tract, which is the main reservoir of GBS in the woman's body. This is because of the proximity between the female gastrointestinal and genitourinary tract. The carriage rate of group B streptococcus in healthy women ranges from 15 – 60% (Mavenyengwa *et al*., 2010). The organism can attach itself to different human cells such as the vaginal epithelial cells, placental membrane cells, respiratory epithelial cells as well as the blood-brain barrier (BBB) endothelium cells (Doran & Nizet, 2004). Infection of the infant with GBS during pregnancy can occur through two different means, i.e. ascending infection and haematogenous infection.

Ascending infection and haematogenous GBS infection

Ascending infection occurs when GBS colonisation ascents from the vagina (main area of colonisation) to the cervix and uterus, causing uterine and amniotic fluid infection, which

eventually lead to infection of the unborn baby as the baby ingest the amniotic fluid. Haematogenous infection occurs when GBS infects the placenta and it is transmitted to the unborn baby through blood circulation in the placenta. Figures 2.2 and 2.3 depict ascending infection and haematogenous GBS infection.

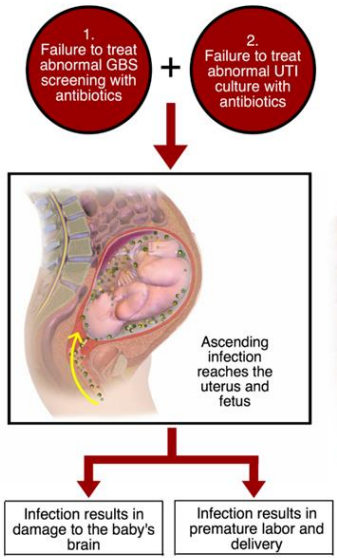

Figure 1.2: Ascending GBS infection

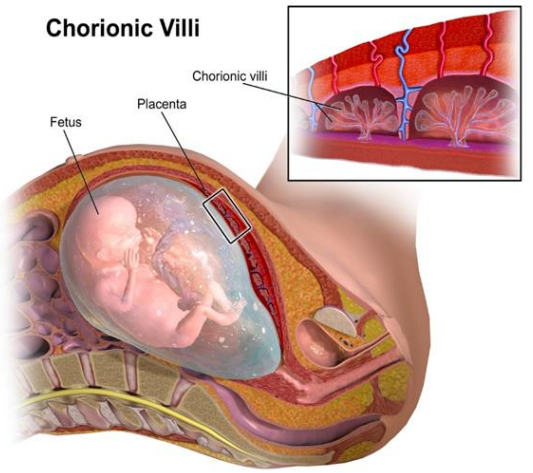

Figure 1.3: Transplacental infection through chorionic villi

Adapted from Reiter & Walsh, 2016

Stages in neonatal GBS infection

Group B streptococcus naturally colonises the female genital tract. In a healthy woman as well as during pregnancy, it attaches itself to the acidic vaginal mucosal cells using adhesion proteins. The bacterium invades the vaginal epithelial cells, causing ascending infection of the uterus and amniotic fluid, resulting in infection of the unborn baby. The bacterium also causes placental infection, thus leading to haematogenous infection of the unborn baby.

In the unborn baby, the bacterium disseminates throughout the blood and invade different epithelial cells such as respiratory epithelial cells and blood brain barrier endothelial cells, leading to bacteraemia, pneumonia and meningitis. The stages in neonatal GBS infection are depicted in Figure 1.4.

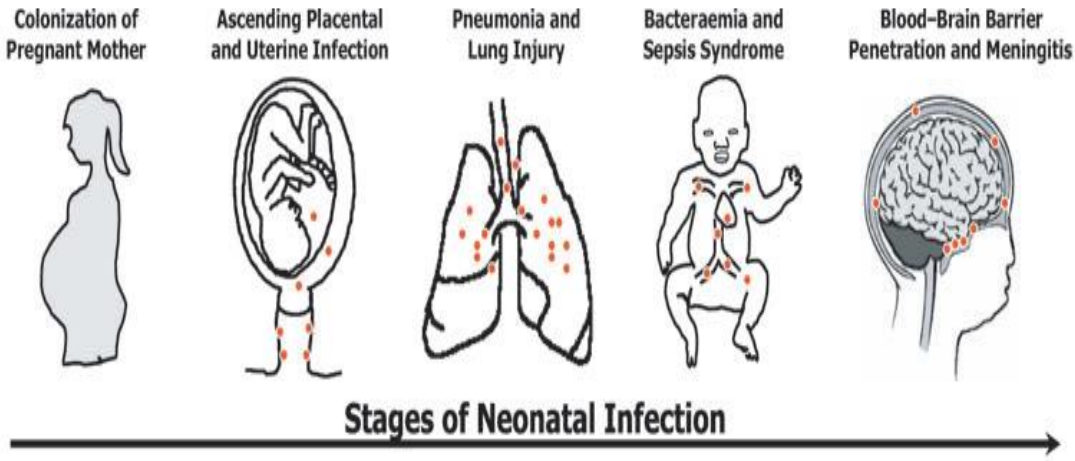

Figure 1.4: Stages in neonatal GBS infection and invasion of the blood-brain barrier

1.2.3 Clinical syndromes of GBS infection in neonates

1.2.4 Early Onset Disease (EOD)

Group B streptococcus infection can be classified in two different clinical syndromes, which are Early Onset Disease and Late Onset Disease (LOD). Early Onset Disease is an infection that presents within the first seven days of the infant's life (between day zero and day seven). This condition is characterized by symptoms of respiratory distress, sepsis, pneumonia and less commonly meningitis. It occurs when the organism is acquired from the mother to the infant during delivery, through the birth canal, or through infection prior to delivery (Alemseged *et al*., 2015; Bidgani *et al*., 2016). The most common GBS capsular types associated with EOD are Ia, II, III and V (le Doare *et al*., 2013).

1.2.4.1 Late Onset Disease (LOD)

Late Onset Disease is defined as an infection that presents between day eight and day ninetieth (third month) of the infant's life (Alemseged *et al*., 2015). This condition is mainly characterized by symptoms of meningitis, bacteraemia and less commonly cellulitis (Alemseged *et al*., 2015; Bidgani *et al*., 2016). The victims of LOD present with meningitis in more than 80% of cases, of which there is approximately 20% mortality and possibility of permanent neurological sequelae (le Doare *et al*., 2013). Since this is a late onset infection, it occurs most commonly due to external factors such as close vicinity between the mother and the newborn baby, through direct contact with other infants who are infected with the

organism or through direct contact with health workers (Narava *et al*., 2014). The most common GBS capsular type widely associated with LOD cases is type III (le Doare *et al*., 2013).

1.2.5 Risk factors for GBS infection

Several studies conducted in African settings have found that vaginal GBS colonisation is associated with risk factors which include social economic factors such as low level of education, genitourinary tract infection during pregnancy, prolonged duration of labour, premature rupture of membranes, low birth weight, presence of GBS in urine and history of abortion, still birth or miscarriages (Joachim *et al*., 2009; Lekala *et al*., 2015; Mitima *et al*., 2014). Human Immuno-Deficiency Virus (HIV) infection also has a significant association with GBS colonisation, however a studies done in Zimbabwe and Malawi to correlate HIV and vaginal GBS colonisation showed no overall correlation (Mavenyengwa *et al*., 2010; Gray *et al*., 2011).

1.2.6 Prevalence of GBS carriage in pregnant women in Sub-Sahara Africa

Group B streptococcus is recognized as a major cause of maternal and neonatal morbidity and mortality in many parts of the world (Joachim *et al*., 2009). Generally, during pregnancy a woman is prone to vaginal GBS colonisation, and the heavier the colonisation, the greater the likelihood of the infant contracting an infection. Approximately 60% of infants born to GBS colonised mothers become colonised with GBS on mucosal surfaces such as oral, nasopharynx, vaginal, anal and skin mucosa (Joachim *et al*., 2009).

The median prevalence of GBS colonisation in pregnant women in African countries was reported at 16% in 2009 (Joachim *et al*., 2009). Table 2.1 summarises the prevalence rate of GBS carriage in pregnant women in various Southern African countries.

Table 1.1: Prevalence of GBS carriage in pregnant women in Southern Africa

Country	Setting/location	Carriage rate (%)
Zimbabwe	Chinhoyi general hospital	31.6% (65/206) in 2000
	Harare - Rutsanana clinic (urban) Guruve (semi-urban) Chitsungo (rural)	Altogether 60.3% (cumulative rate) (470/780) colonisation (highest rate observed in rural, followed by semi-urban and urban) in 2009
South Africa	Soweto, Johannesburg	28.7% at 31-37 weeks 28.4% at 37 + weeks
	Dr George Mukhari teaching hospital Pretoria (rural, semi and urban)	Altogether 30.9% (128/413) in 2012
Malawi	Queen Elizabeth Central Hospital	21.2% (394/1857) in 2010
Mozambique	Maputo (urban)	1.8% (2/113) in 2008
Namibia	Windhoek (urban)	13.6% (58/588)

Adapted from Moyo *et al*., 2000; Mavenyengwa *et al*., 2009; Chukwu *et al*., 2015; Monyama *et al*., 2016; Gray *et al*., 2011; de Steenwinkel *et al*., 2008 & Engelbrecht *et al*., 2016

Differences in positive rates found in various African settings can be attributed to variations in habitats, socio-demographic factors, differences in isolation media used, recovery from different sites and specimens that were used in different studies.

In the Mozambican study, rectovaginal swabs were inoculated in Todd-Hewitt broth with only gentamycin (8 µg/ml of agar) and the broth was subcultured onto human blood agar following 24 hours of incubation. The use of gentamycin alone without nalidixic acid can cause overgrowth of enterobacteriaceae organisms over GBS. Furthermore, the use of human blood in preparation of blood agar might have influenced the results as human blood may contain inhibitory substances against the growth of GBS (de Steenwinkel *et al*., 2008).

1.2.7 GBS polysaccharide capsular types and their geographical distributions

Group B streptococcus is classified into ten common capsular types based on the polysaccharide capsular antigens on the surface of the organism. These are Ia, Ib, II, III, IV, V, VI, VII, VIII, IX (Wang *et al*., 2015; Teatero *et al*., 2014). Capsular type distribution differs significantly with geographical region and ethnic origin of the population. Generally, capsular type Ia, III and IV are the most predominant capsular types in most parts of the world, except for Japan where type VI and VII are more common (Chukwu *et al*., 2015).

In Southern Brazil, a study was conducted to phenotypically characterize GBS isolates from inpatients and outpatients attending the clinical hospital of the federal university of Parana, which found type IV to be the most predominant cause of infections (Palmeiro *et al*., 2010).

In China, a study was conducted to determine the capsular type distribution of invasive GBS isolates recovered from neonates, which found type III to be the predominant type accounting for 85% of all cases, followed by Ia (7.3%), Ib (5%) and V (2.5%) (Wang *et al*., 2015). In Australia, GBS capsular type Ia, II and V are the leading causes of invasive infection (Zhao *et al*., 2008).

A study conducted in Gabon, Central Africa to determine maternal GBS capsular type distribution concluded that almost a third (30.3%) of isolates were capsular type V, followed by III (27.5%), Ib (22.9%), Ia (12.8%) and II (6.4%). From the same study, none of capsular type IV, VI, VII, XIII or XI were identified (Belard *et al*., 2015).

A study conducted to determine the antigenic distribution of *S. agalactiae* isolates from pregnant women at Garankuwa hospital in South Africa revealed that capsular type III was the most common isolated type (29.7%), followed by Ia (25.6%), II (15.6%) IV (8.6%), V (10.9%) and Ib (8.6%) (Chukwu *et al*., 2015). This means that the most common GBS capsular types associated with this population are III, Ia and II.

Another South African study conducted on pregnant mothers attending prenatal clinics in Soweto concluded that capsular type III was more associated with persistent colonisation throughout the study (29%) than Ia (18%) and V (6%) (Kwatra *et al*., 2014). In a Zimbabwean study, the following capsular types were identified as follows: la, lb, II, III and V at 15.7%, 11.6%, 8.3%, 38.8% and 24.0% respectively (Mavenyengwa *et al*., 2010).

1.2.8 Isolation and identification of GBS

The CDC recommends prevention of invasive neonatal GBS infection through prenatal culture-based screening of rectovaginal swabs. This screening is recommended in all pregnant women at 35 – 37 weeks of gestation, using a vaginal and rectal swab from each pregnant woman (CDC, 2010; Imperi *et al*., 2011; Alp *et al*., 2016). Various identification methods are available to confirm beta haemolytic streptococcus as *S. agalactiae*.

1.2.8.1 Cultural based isolation of GBS

Isolation of GBS involves direct inoculation of a lower vaginal and rectal swab onto 5% sheep blood agar (SBA) and into Todd-Hewitt (TH) enrichment broth. Both media can be supplemented with colistin, gentamycin (8 μg/ml) or nalidixic acid (15 μg/ml) to increase their selectivity towards GBS.

Granada agar is used as a differential medium for group B streptococcus. This medium possesses improved stability and selectivity (BD, 2013). This medium inhibits the growth of staphylococci, gram negative rods and strict anaerobes. Other streptococci such as enterococci growth without inhibition, however they grow in colourless to gray or gray-blue colonies, unlike *S. agalactiae* which grows in orange colonies on Granada agar. Occasional GBS strains which are non-haemolytic however do not grow in orange colonies (BD, 2013). Therefore, it is essential that sheep blood agar is used together with Granada agar in the isolation of GBS.

When only traditional culture (direct agar plating) is used without additional selective enrichment broth, about 50% of women who are GBS colonised might yield false negative culture results (CDC, 2010). On blood agar, GBS grows in greyish-white, translucent, mucous colonies surrounded by a small zone or bigger zone of β-haemolysis.

1.2.8.2 Identification of GBS using traditional methods

Once suspicious GBS growth is encountered, gram stain, catalase, bacitracin sensitivity and Christie, Atkins, Munch and Petersen (CAMP) test can be performed to further identify the organism (Winn *et al*., 2006).

Gram stain

This test is used to classify bacteria by their gram reaction and morphology. The iodine-crystal complex is easily removed from more permeable cell wall of gram negative bacteria compared to less permeable cell wall of gram positive bacteria (Winn *et al*., 2006). Gram negative bacteria destain with acid alcohol and counterstain with carbol fuchsin stain, whereas gram positive bacteria retain the crystal violet-iodine complex (Winn *et al*., 2006).

Catalase test

The catalase test is used to differentiate between bacterial organisms that produce an enzyme catalase, such as staphylococci, from non-catalase producing bacteria such as streptococci (Winn *et al*., 2006). *S. agalactiae* is catalase negative (Winn *et al*., 2006).

Bacitracin sensitivity test

The bacitracin test identifies whether beta haemolytic colonies are Group A, (*Streptococcus pyogenes* (*S. pyogenes*)) or Group B streptococci (Winn *et al*., 2006). This test is based on the ability of the organism to resist bacitracin. *S. agalactiae* is bacitracin resistant (Winn *et al*., 2006).

CAMP test

The CAMP test presumptively identifies *S. agalactiae* based on a synergistic reaction between its beta haemolysin, (an extracellular diffusible protein called CAMP factor that it produces), and the staphylococcal beta lysin toxin produced by *Staphylococcus aureus* (*S. aureus*) (Winn *et al*., 2006). The presence of CAMP factor in GBS relies on the existence of the *cfb* gene which encodes for the CAMP protein. Although almost all GBS strains are CAMP positive, CAMP activity may be decreased in certain isolates and this may affect GBS identification.

The CAMP factor encoding gene *cfb* is present in almost all GBS isolates, however CAMP factor negative isolates have been described but may be rare (Savini *et al*., 2016). CAMP negative GBS isolates containing a normal sized *cfb* gene have also been described, CAMP negative reaction of such isolates could rely on reduced expression of the CAMP protein (Savini *et al*., 2016).

This test is performed by streaking a known beta haemolytic *S. aureus* strain across a 5% sheep blood agar, followed by inoculating the test organism perpendicularly proximal to the *S. aureus* streak (Winn *et al*., 2006). The Group B streptococcal beta haemolysin toxin interacts with the staphylococcal beta lysin in the presence of sheep erythrocytes, producing enhanced arrow-head shaped haemolysis where the two organisms meet, indicating a positive reaction (Savini *et al*., 2016). This is used for presumptive identification for *S. agalactiae*. *S. pyogenes* can be used as a negative control (*Winn et al*., 2006).

1.2.8.3 Identification of GBS using polymerase chain reaction (PCR)

Polymerase chain reaction is a molecular technique based on the amplification of a single stranded deoxyribonucleic acid (DNA) template into million copies. This process occurs with the help of an enzyme DNA *Taq* polymerase, which synthesises a new DNA strand complementary to the original DNA template through addition of nucleotides (Mohini & Deshpande, 2011).

Confirmation of GBS can be done by amplifying and detecting a target gene which is specific for GBS, namely the *scp*B gene. The *scp*B gene encodes for the 5Ca peptidase, which is an adhesin protein and one of the virulence factors for GBS.

Previously, the G5a peptidase genes were only found in Group B streptococcus (*scp*B gene), Group A streptococcus (*scp*A gene) and Group G streptococcus (*scp*G gene) (Dmitriev *et al*., 2004). The *scp*B gene has a 51-base pair (bp) deletion at the 3' end of the gene, which makes it different from the rest. In this study, detection of the *scp*B gene was considered as a specific diagnostic marker for *S. agalactiae*. The *scp*A and *scp*G genes have 306 base pairs, while the *scp*B gene only has 255 base pairs (51 bp difference) (Dmitriev *et al*., 2004).

1.2.9 Antimicrobial susceptibility patterns

Antimicrobial susceptibility patterns of GBS might vary from region to region due to differences in GBS capsular types present in such areas (Alemseged *et al*., 2015). Most studies done in Africa have shown uniformity in sensitivity of GBS to penicillin and ampicillin, the recommended drugs of choice against GBS.

In a study conducted in Ayder referral hospital and Mekelle health centre, Mekelle, Northern Ethiopia, GBS was reportedly 100% susceptible to penicillin, ampicillin, erythromycin, gentamycin and vancomycin (Alemseged *et al*., 2015). In the same study, GBS isolates were found to have intermediate susceptibility to chloramphenicol by 42.1%, ceftriaxone by 26.3%, ciprofloxacin by 21.1% and novofloxacin by 15.8%.

In a similar study conducted in Ghandi memorial hospital and Tikur Anbessa, Addis Ababa, Ethiopia, GBS was found to be 55% susceptible to penicillin, 91% sensitive to ampicillin and all isolates except one were susceptible to erythromycin (Woldu *et al*., 2014). Increased

resistance in this area was attributable to a wide non-prescription use of penicillin due to weak drug control system (Woldu *et al*., 2014).

A study conducted in Gabon, Central Africa to determine capsular type distribution and antimicrobial susceptibility of GBS in pregnant women found that all GBS isolates were susceptible to clindamycin, cefotaxime, linezolid, vancomycin, benzylpenicillin, cefalotin and high level gentamycin (HLG) (Belard *et al*., 2015). Fourteen isolates were however found to have intermediary susceptibility to erythromycin, and no inducible clindamycin resistance was found (Belard *et al*., 2015).

In South Africa, a study conducted to assess the antimicrobial resistance of *S. agalactiae* isolated from pregnant women in Garankuwa found that all strains were 100% susceptible to penicillin, ampicillin, vancomycin and high level gentamycin (Bolukaoto *et al*., 2015). The same study however found some strains to be resistant to erythromycin, clindamycin, tetracycline, chloramphenicol and ciprofloxacin by 21.1%, 17.2%, 94.5%, 24.9% and 18.6% respectively.

In a similar study conducted in Soweto, South Africa to determine the risk of GBS sepsis in infants exposed to HIV, it was found that 385 of 389 isolates were penicillin sensitive, and macrolide resistance was observed in 15 (5.5%) of 273 isolates (Cutland *et al*., 2015).

In Tanzania, a study conducted on maternal and neonatal GBS colonisation at Muhimbili national hospital in Dar es Salaam found all GBS isolates to be 100% sensitive to vancomycin and ampicillin, 90 – 100% sensitive to penicillin and ciprofloxacin and 80 – 90% sensitive to clindamycin, erythromycin, cotrimoxazole and ceftriaxone (Joachim *et al*., 2009).

In Windhoek, Namibia, GBS' resistance was observed as follows in 2016: contrimoxazole 6% (7/117) tetracycline 94.9% (111/117) erythromycin 11.1% (13/117), clindamycin 8.5% (10/117), inducible clindamycin resistance 5.1% (6/117) and cefoxitin 100% sensitive (Engelbrecht *et al*., 2016).

Inducible clindamycin resistance is defined as a resistance mechanism that occurs due to stimulation and mutation of genes because of exposure to inciting factors (Mahon *et al*., 2007). This mechanism of resistance occurs when the bacterial strains possess an inducible gene, which has high potential of developing spontaneous mutations over the course of clindamycin treatment with a second drug erythromycin.

An *erm* gene is a ribosome methylase which encodes for methylation of the 23S ribosomal Ribonucleic acid (rRNA), and it is minimally expressed. Erythromycin induces the production of this methylase, thus causing resistance to erythromycin. Methylation of the 23S rRNA causes mutations in the 23S rRNA, which causes resistance to clindamycin. However, the *msrA* gene encodes for the efflux mechanism, which results in resistance to erythromycin and susceptibility to clindamycin. Therefore, in the presence of erythromycin, an organism might develop resistance to clindamycin as well (Mahon *et al*., 2007).

Inducible clindamycin resistance is confirmed by performing a D-zone test using a disk diffusion method. The test is performed by placing an erythromycin disc near a clindamycin disc (15 mm apart) on blood agar that has been inoculated with the test isolate. The plate is then incubated for 24 hours at 37°C.

The flattening of the zone of inhibition around the clindamycin disc proximal to the erythromycin disc producing a zone of inhibition shaped like the letter D, is considered a positive result and indicates that erythromycin has induced clindamycin resistance (Levin *et al*., 2005). Figure 2.5 illustrates a negative and positive inducible clindamycin resistance, whereby A represents an erythromycin disc and B represents a clindamycin disc.

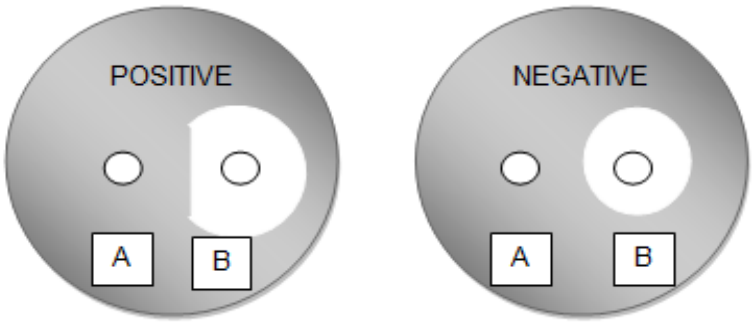

Figure 1.5: Inducible clindamycin resistance

Adapted from Levin *et al*., 2005

Principle of Vitek 2

The Vitek 2 (bioMerieux, Midrand, South Africa) system is an automated microbiology system which utilizes a growth-based method for identification and sensitivity testing of bacteria. This system uses colorimetric reagent cards that are incubated and interpreted automatically (bioMerieux, 2014). Sensitivity testing is based on a growth-based method at antimicrobial

intervals to determine the minimum inhibitory concentration that completely inhibits bacterial growth.

1.2.10 Mechanisms of antimicrobial resistance

1.2.10.1 Genetic basis of antimicrobial resistance

Genetic based resistance occurs when a bacterial organism develops resistance against an antibiotic due to modification or alteration of the bacteria's genetic make-up. The two common genetic strategies through which bacteria adapt antimicrobial attack are: mutations in genes associated with the mechanism of action of the antimicrobial agents and acquisition of foreign DNA coding for resistance, through horizontal gene transfer (Munita & Arias, 2016).

Mutational resistance

Susceptible bacteria can develop mutations in genes that affect the activity of the drug, resulting in survival of the bacteria in the presence of the antimicrobial agent (Munita & Arias, 2016). These mutations alter the antibiotic action via the following mechanisms: modification or alteration of the antimicrobial target site, decrease in drug uptake, activation of efflux mechanism to extrude the antibiotics and changes in metabolic pathways (Munita & Arias, 2016).

Horizontal gene transfer

Horizontal gene transfer refers to acquisition of foreign DNA material from the environment, causing alteration of the genetic make-up and consequently affecting the activity of antimicrobial agents (Munita & Arias, 2016). Acquisition of external DNA material in bacteria occurs through transformation, transduction and conjugation (Munita & Arias, 2016).

1.2.10.2 Mechanistic basis of antimicrobial resistance

Modification of the antibiotic molecule

Various bacterial organisms resist the presence of antibiotics by producing enzymes which inactivate the drug by adding molecules to it, thus preventing the antibiotic from interacting with its target site. Common enzyme catalysed biochemical reactions that render antibiotics inability to exert their effects include acetylation (aminoglycosides, chloramphenicol, streptogramins), phosphorylation (aminoglycosides, chloramphenicol) and adenylation

(aminoglycosides, lincosamides). All these biochemical reactions decrease the avidity of the antibiotics to their target sites (Munita & Arias, 2016).

Furthermore, some bacteria produce enzymes that destroy the antimicrobial compounds completely. This includes organisms producing β-lactamases, which destroy the amide bond of the β-lactam ring, rendering the antibiotic ineffectiveness (Munita & Arias, 2016).

Decreased antibiotic penetration and efflux

Bacteria have developed mechanisms of preventing the intracellular influx of antimicrobial agents to reach their target sites (Munita & Arias, 2016). This happens because of changes in types of porins expressed, changes in the level of porin expression on bacterial surfaces and impairment of porin function, which reduces drug uptake (Munita & Arias, 2016).Moreover, certain bacterial organisms are capable of producing efflux pumps which extrude toxic compounds out of bacterial cells. This process is mediated by *mef* genes which encode for efflux pumps, and commonly results in resistance to macrolides (Munita & Arias, 2016).

Changes in antibiotic target sites

This mechanism allows bacteria to alter the antimicrobial target site by either protecting it or modifying it, thus decreasing its affinity for the antibiotic molecule (Munita & Arias, 2016). This mechanism commonly results in resistance to tetracycline. The genes *tet*(M) and *tet*(O) interact with the ribosome and dislodge tetracycline from its binding site. This interaction alters the ribosomal conformation, preventing rebinding of tetracycline (Munita & Arias, 2016).

Modification of antibiotic target sites can take place through point mutations in the genes encoding the target sites, enzymatic alteration of binding sites and replacement of target sites, thus decreasing the target site's affinity for the antibiotic (Munita & Arias, 2016).

The most common mechanisms of antimicrobial resistance demonstrated by GBS include mediation of efflux pumps and ribosomal alteration or interaction. Genes encoding for resistance commonly reported in GBS include *ermB, ermA, ermTR, mefA, mefE, aphA-3, aad-6, tet(M), tet(O), int-Tn* and *mreA* (Zeng *et al*., 2006). Table 2.2 illustrates the genes that encode for GBS's resistance against antibiotics.

Table 1.2: Genes that encode for GBS' resistance against antibiotics

Genes	Functions of the genes	Antibiotics affected
tet(M) and *tet*(O)	They are elongation factor proteins and they interact with the ribosome and dislodge the antibiotic tetracycline	Tetracycline
erm(B) and *erm*(A/TR)	They encode the 23S rRNA methylase enzyme which alters the antibiotic binding site thus causing resistance of the antibiotic	Constitutive/inducible cross resistance to erythromycin, clindamycin and streptogramin B antibiotics
mef(A/E)	They mediate an efflux pump	Erythromycin
linB	Codes for ribosomal translocation	Clindamycin

Adapted from Bolukaoto *et al*., 2015; Gygax *et al*., 2006 & Zeng *et al*., 2006

1.2.11 Intrapartum Antibiotic Prophylaxis (IAP)

Penicillin or ampicillin are the drugs of choice for intrapartum antibiotic prophylaxis in GBS positive pregnant women (CDC, 2010). However, ampicillin is less commonly used because of the level of resistance existing in other bacteria against this antibiotic (Engelbrecht *et al*., 2016). It is however preferred due to its broad spectrum and it is safer to use during pregnancy.

Before 2002, clindamycin and erythromycin were the preferable antibiotics for prophylaxis in pregnant women who are allergic to penicillin (Engelbrecht *et al*., 2016). On the contrary, data on the ability of clindamycin, erythromycin and vancomycin to reach bactericidal levels in the foetal circulation and amniotic fluid are very limited. Available data suggest that erythromycin and clindamycin provided to the pregnant women do not reach foetal tissues reliably (CDC, 2010). In addition, erythromycin treatment for GBS positive pregnant mothers does not decrease the chances of preterm delivery (Engelbrecht *et al*., 2016).

Due to accelerated use of clindamycin and erythromycin, it has been reported that there is an elevation in resistance to these drugs amongst GBS isolates (Engelbrecht *et al*., 2016). The emergence of resistance to erythromycin and clindamycin amongst Group B streptococcus has prompted the review of Centres for Diseases Control and Prevention guidelines for preventing perinatal GBS disease. The new guidelines require assessment of the history of penicillin allergic mothers, to determine their risk of a fatal allergic reaction against penicillin.

Pregnant women who are allergic to penicillin not classified as type 1 hypersensitivity reactions or who have no history of anaphylaxis, angioedema or respiratory distress following administration of penicillin or cephalosporin should receive cefazolin (CDC, 2010). This is because they are known to present with a low risk to cephalosporins.

Penicillin-allergic women at high risk of anaphylaxis should receive clindamycin if their isolate is susceptible to clindamycin and erythromycin (CDC, 2010). If the isolate is sensitive to clindamycin but resistant to erythromycin, clindamycin may be used if testing for inducible clindamycin resistance is negative (CDC, 2010).

Penicillin-allergic women who are at high risk of anaphylaxis and whose isolates exhibit inducible clindamycin resistance or whose isolates' susceptibilities to both agents are unknown, should receive vancomycin (CDC, 2010). Figure 2.4 summarises the four categories used for the selection of antibiotics for intrapartum antibiotic prophylaxis, as recommended by CDC (CDC, 2010).

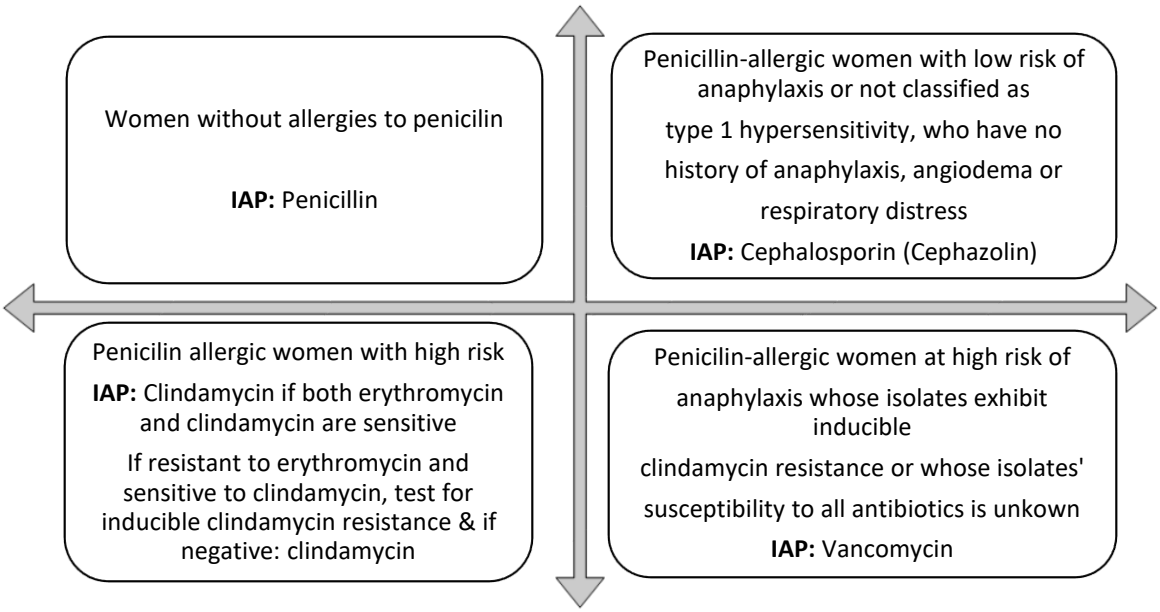

Figure 1.6: The four categories used for selection of antibiotics for IAP

Adapted from CDC, 2010

The CDC has also developed indications and contra-indications of intrapartum antibiotic prophylaxis to prevent early-onset Group B streptococcal disease. Table 2.3 summarises the indications and contra-indications of intrapartum antibiotic prophylaxis.

Table 1.3: Indications and contra-indications for intrapartum antibiotic prophylaxis to prevent early-onset Group B streptococcal disease

Intrapartum GBS prophylaxis indicated	Intrapartum GBS prophylaxis not indicated

• Previous infant born with GBS infection
• GBS bacteriuria in current pregnancy (if vaginal delivery only).
• Positive GBS culture screening at 35 – 37 weeks during current pregnancy.
• Unknown GBS status at the onset of labour and any of the following:
  ○ Delivery at <37 weeks of gestation
  ○ Amniotic membrane rupture ≥18 hours
  ○ Intrapartum temperature ≥38.0°C
	• GBS colonization in previous pregnancy
	• GBS bacteriuria in previous pregnancy
	• Negative vaginal and rectal GBS culture screening at 35 – 37 weeks during the current pregnancy, regardless of intrapartum risk factors.
	• Caesarean delivery performed before onset of labour on a woman with intact amniotic membranes, regardless of GBS colonization status or gestational age.

Adapted from CDC, 2010

1.2.12 Statement of the research problem

Group B streptococcus causes nearly over 50% of mortality rate in neonates and preterm infants worldwide (Heelan *et al*., 2004). It equally colonises 20 – 40% of healthy women, consequently affecting 50 – 70% of infants (Doran & Nizet, 2004). A GBS positive colonisation rate was reported at 13.6% in pregnant women in Windhoek in the year 2016 (Engelbrecht *et al*., 2016).

Group B streptococcus' presence in the vagina during pregnancy is associated with maternal infections such as endometritis, chorioamnionitis in the pregnant mother (Mitima *et al*., 2014). These infections can cause spontaneous abortion, premature delivery, still births, mental retardation and hearing loss (Alemseged *et al*., 2015). It is also associated with neonatal infections such as meningitis, sepsis and pneumonia (Mitima *et al*., 2014).

Although many studies have shown that GBS is uniformly susceptible to penicillin and ampicillin, the emergence of increasing minimum inhibitory concentration (MIC) and intermediate sensitivity results to penicillin have been reported in some areas. In a study

conducted in Osaka, Japan, 18% intermediate susceptibility and 1% resistance to penicillin was reported in GBS in the year 2003 (Morikawa *et al*., 2003).

There is limited information regarding the prevalence of GBS carriage in pregnant women from rural areas in Namibia, their antimicrobial susceptibility patterns, their capsular type distribution as well as their gene based resistance. This study determined the prevalence and molecular characteristics of GBS isolates from pregnant women in the selected rural areas in Namibia. This yielded baseline information regarding the status of GBS colonisation in pregnant women who attended antenatal screening at Onandjokwe, Eenhana and Okongo state hospitals from May to September 2018.

1.2.13 Research objectives

Main objective

The main objective of this study was to investigate the prevalence of Group B streptococcus in pregnant women from hospitals in Oshikoto and Ohangwena regions of Namibia. In addition, capsular types, antimicrobial susceptibility patterns and gene based resistance of the isolates were investigated.

Specific objectives

1) To determine the prevalence of GBS carriage in pregnant women from hospitals in Oshikoto and Ohangwena region.
2) To determine the capsular types of GBS isolates in pregnant women from hospitals in Oshikoto and Ohangwena region.
3) To determine the antimicrobial sensitivity patterns and gene based resistance of the isolates.

CHAPTER 2: METHODOLOGY

2. METHODOLOGY

2.1 Study design

This was a descriptive cross sectional study which gathered descriptive data such as socio-economic, demographic characteristics and obstetric factors for the selected population. This study was based on quantitative approach, as it involved calculation of Group B streptococcus' prevalence rate.

2.2 Study population

The population of this study comprised of pregnant women above 35 weeks of gestation, who attended antenatal screening at Onandjokwe, Eenhana and Okongo state hospitals between May and September 2018. Due to limited availability of participants, a gestational range was changed from 35 – 37 weeks to 35 weeks of gestation and above.

2.2.1 Inclusion criteria

Pregnant women at 35 weeks of gestation on onwards were involved in the study.

2.2.2 Exclusion criteria

Pregnant women who took antibiotics two weeks prior to contact were excluded, as well as those aged below 16 years.

2.2.3 Study setting

This study was conducted in Onandjokwe, Eenhana and Okongo state hospitals. Specimens were collected from women attending antenatal screening at the mentioned hospitals, and were analysed at the Namibia University of Science and Technology, medical laboratory sciences department.

2.3 Sample size

The sample size was calculated based on the prevalence rate of GBS colonisation in pregnant women found in a study conducted in Windhoek, which was 13.6% (Engelbrecht *et al*., 2016). The sample size was calculated using the following formula:

$$n = \left(\frac{(Z)^2 \times p(1-p)}{d^2}\right) \qquad n = \left(\frac{(1.96)^2 \times 0.136(1-0.136)}{(0.05)^2}\right) \qquad n = \left(\frac{3.8416 \times 0.136(0.864)}{0.0025}\right)$$

n = 181 participants

Where: n = sample size

Z = value of 95% confidence interval (1.96)

P = proportion of GBS colonisation (13.6%) = 0.136

d = margin of error (5%) = 0.05

A ten percent (10%) non-response rate (i.e. $\frac{10}{100}$ × 181 = 18.1) was added to the sample size. A total of 200 participants (181 + 18.1) were recruited.

2.4 Sampling technique

A convenient sampling technique was used in this study. This is a non-probability sampling technique which includes selection of participants based on availability. This sampling technique was suitable for this study as it allows inclusion of participants based on their availability and voluntary participation.

The study aimed at recruiting 50 participants from each of two hospitals in Oshikoto region and 50 from each of two hospitals in Ohangwena region. However, due to lack of participation, this could not be achieved. Participants were therefore recruited based on their availability at each hospital, until the total sample size was obtained.

2.5 Materials and Methods

2.5.1 Specimen collection and transportation

Prior to specimen collection, each participant signed a written consent to participate in the study. The consent form explained the purpose and benefits of the study, and it was available in relevant local languages.

A lower vaginal and rectal swab was aseptically collected from each participant by registered nurses using sterile cotton-tipped swabs. For each participant, a lower vaginal swab was

collected by inserting and brushing a sterile swab 2cm into the vagina, periurethral area and labia. A rectal swab was collected by inserting a sterile swab 1cm into the anus, rubbing the wall of the anal canal as recommended by CDC (CDC, 2010). The swabs were inserted into Amie's transport medium for preservation of bacteria. Each specimen was labelled with a unique identification. Specimens were transported at 2 – 8°C to the laboratory.

2.5.2 Preparation of sheep blood agar (SBA)

Before the preparation of sheep blood agar, fresh sheep blood was acquired from the local veterinary laboratory. Molten Columbia agar was prepared by dissolving 39g of Columbia agar base in 1 000ml of distilled water, followed by autoclaving at 121°C for 15 minutes. The agar was left to cool down to 50°C, followed by the addition of 50ml of sterile sheep blood. The media was gently mixed by swirling and was poured into sterile petri dishes. Plates were then labelled as SBA with the date of preparation, lot number, batch number and expiry date.

Quality control was performed on the plates by inoculating 5% of the prepared SBA plates with *S. agalactiae* ATCC 12403, followed by incubation at 37°C for 24 hours. Sterility check was performed on the plates by incubating 5% of un-inoculated SBA plates from the same batch at 37°C for 24 hours. The rest of the plates were stored at 2 – 8°C (Engelbrecht *et al*., 2015).

After 24 hours of incubation, quality control and sterility plates were examined for growth. Plates were deemed acceptable for use only if the 5% quality control plates inoculated with *S. agalactiae* ATCC 12403 presented with grey whitish β-haemolytic colonies and the 5% uninoculated plates presented with no growth. The expiry date of SBA plates was 3 months following the date of preparation.

2.5.3 Preparation of Sheep blood agar with colistin and nalidixic acid (BCNA)

Preparation of sheep blood with colistin and nalidixic acid was done using a method adapted from Engelbrecht *et al*., 2015. Molten agar was prepared by dissolving 43g of Columbia CNA agar base in 980ml of distilled water, followed by autoclaving at 121°C for 15 minutes. Before solidifying, 55ml of sterile sheep blood was aseptically added to the molten agar. The media was mixed gently by swirling, poured into sterile petri dishes and allowed to solidify. After solidifying, the plates were labelled as BCNA, with the date of preparation, lot number, batch number and expiry date.

Quality control was performed on the plates by inoculating 5% of the prepared BCNA plates with *S. agalactiae* ATCC 12403, followed by incubation at 37°C for 24 hours. Sterility check was performed on the plates by incubating 5% of uninoculated BCNA plates from each batch at 37°C for 24 hours. The rest of the plates were stored at 2 – 8°C (Engelbrecht *et al*., 2015). This was done once for every batch.

After 24 hours of incubation, quality control and sterility plates were examined for growth. Plates were accepted for use only if the 5% quality control plates inoculated with *S. agalactiae* ATCC 12403 presented with grey whitish β-haemolytic colonies and the 5% uninoculated plates presented with no growth. The expiry date of BCNA plates was 3 months following the date of preparation.

2.5.4 Preparation of Todd-Hewitt Broth

Todd-Hewitt broth was used in this study to enhance the growth of beta-haemolytic streptococci. Todd-Hewitt broth's selectivity for GBS was enhanced by adding colistin and nalidixic acid, which inhibit the growth of commensals of the genital and gastrointestinal tract (Engelbrecht *et al*., 2015).

Todd-Hewitt broth was prepared using a modified method described by Engelbrecht *et al*., 2015. In this method, 13g of Todd-Hewitt broth powder was dissolved in 980ml of distilled water, followed by autoclaving at 121°C for 15 minutes. About 0.008g (8µg/ml of agar) of colistin and 0.015g (15µg/ml of agar) of nalidixic acid powder were dissolved in 20ml of distilled water. Dissolved antibiotics were added to the broth cooled to room temperature (Engelbrecht *et al*., 2015). The broth was gently mixed and dispensed in 3ml aliquots into sterile 5ml glass bottles. The bottles were labelled with the broth name, preparation date, lot number, batch number and expiry date.

Quality control was performed on the broth by inoculating 5% of bottles with *S. agalactiae* ATCC 12403, followed by incubation at 37°C for 24 hours. Sterility check was performed on the broth by incubating 5% of uninoculated bottles from the same batch at 37°C for 24 hours. The rest of the bottles were stored at 2 – 8°C (Engelbrecht *et al*., 2015).

After 24 hours of incubation, quality control and sterility bottles were examined for growth. The broth was acceptable for use only if the bottles inoculated with *S. agalactiae* ATCC 12403

presented with turbidity and those uninoculated presented with no turbidity. The expiry date of the broth was 3 months following the date of preparation.

2.5.5 Preparation of glycerol broth

Glycerol broth was used in this study for the preservation of frozen GBS isolates. It was prepared by adding 10ml of glycerol to 40ml of Todd-Hewitt broth to make up a total of 50ml of broth. The broth was labelled as glycerol broth with date of preparation, lot number and expiry date, and it was kept at 2 – 8°C. Each presumptive GBS isolate was then inoculated and frozen in 1ml of glycerol broth dispensed in a 2ml corning cryogenic vial.

2.5.6 Preparation of nutrient agar

Nutrient agar was prepared by dissolving 28g of nutrient agar base in 1 000ml of distilled water, followed by autoclaving at 121°C for 15 minutes. The media was left to cool after which it was poured in sterile petri dishes and left to solidify. The plates were then labelled as nutrient agar with the date of preparation, lot number, batch number and expiry date.

2.5.7 Specimen culture

Each swab was inoculated directly onto a BCNA plate, followed by inoculation into Todd-Hewitt broth supplemented with colistin and nalidixic acid. The plates and broths were incubated at 37°C in CO_2 for 24 hours. After incubation, BCNA plates were examined for presence of large, whitish-grey and translucent colonies with a narrow zone of β-haemolysis while the Todd-Hewitt broths were subcultured onto BCNA agar and examined the following day.

The subcultured BCNA plates were incubated at 37°C in CO_2 for 24 hours. After incubation, the plates were examined for presumptive GBS growth. CAMP test was performed to presumptively identify the organism as *S. agalactiae* (Chaiwarith *et al*., 2011).

2.5.8 Presumptive identification of *S. agalactiae* using CAMP test

CAMP test is used to presumptively identify *S. agalactiae.* This test was performed by streaking a known β-haemolytic *S. aureus* clinical isolate across the 5% sheep blood agar, followed by inoculating the test organism perpendicularly to the *S. aureus* streak, making sure

the two inoculums do not meet (Cheesbrough, 2006). *S. agalactiae* ATCC 12403 was used as a positive control and *Streptococcus pyogenes* ATCC 1244 was used as negative control.

2.5.9 Preservation of presumptive *S. agalactiae* isolates

All CAMP positive colonies were subcultured on sheep blood agar for pure cultures. Pure growth of CAMP positive colonies was then harvested from all plates and preserved in glycerol broth in 2ml cryogenic vials. The broths were then frozen at – 80°C.

2.5.10 Confirmation of *S. agalactiae* using polymerase chain reaction

All CAMP positive GBS isolates were confirmed using PCR and *S. agalactiae* ATCC 12403 was included as a positive control. In this method, a virulence gene *scp*B was amplified as a target gene through real time PCR as previously described by Dmitriev *et al*., 2004.

2.5.10.1 DNA extraction

Before DNA extraction, glycerol preserved presumptive GBS isolates were thawed and streaked on nutrient agar for single colonies, followed by incubation at 37°C for 24 hours. A boiling method was used for DNA extraction as described by Gygax *et al*., 2006. In this method, 200µl of nuclease free water was added to 5ml eppendorf tubes labelled 1 to 18, each number representing an isolate. An extra eppendorf tube was added for the positive control.

Pure culture of presumptive isolates were harvested from the nutrient agar and emulsified in the respective eppendorf tube using a sterile plastic loop. The suspensions were then heated at 100°C in the heating block for 15 minutes. Following the heating, the tubes were centrifuged at 8 000 revolutions per minute (rpm) for 10 minutes. The supernatant containing DNA was transferred to a clean labelled eppendorf tube, and the sediment containing debris was discarded.

2.5.10.2 Preparation of PCR primers

The following primers for the *scp*B gene were used: forward primer 5’ – ACAACGGAAGGCGCTACTGTTC – 3’ and reverse primer 5’ – ACCTGGTGTTTGACCTGAACTA – 3’. The primer’s stock solution of 100µM and working solution of 10µM were prepared as per the manufacturer’s instructions (Inqaba Biotechnical Industries, Pretoria, South Africa).

2.5.10.3 Real time polymerase chain reaction

A mixture of PCR components was prepared by transferring nuclease free water, PCR master mix, forward and reverse primer as shown in the Table 2.1.

Table 2.1: Preparation of real time PCR components for GBS confirmation

PCR component	Volume
PCR master mix with colour	240µl
Nuclease free water	210µl
Forward primer	20µl
Reverse primer	20µl
Total volume	490µl

The solution was mixed gently and dispensed in 24.5µl aliquots into 20 PCR tubes labelled from 1 – 18, with a positive and negative control included. Exactly 5µl of the DNA, positive control and negative control (nuclease free water) was added to each respective 0.2ml PCR tube, followed by gentle mixing. The PCR tubes were then loaded in the thermal cycler (BioRad, Johannesburg, South Africa) and conditions illustrated in the Table 2.2 were applied.

Table 2.2: Real time PCR conditions for GBS confirmation

PCR cycles	Phase	Temperature	Time
1x	Initial denaturation	94 °C	4 minutes
	Denaturation	93 °C	1 minute
35x	Annealing	57.6 °C	1 minute
	Elongation	72 °C	1 minute
1x	Prolonged elongation	72 °C	7 minutes
Dwell	Hold	4 °C	Infinity

2.5.10.4 Preparation of 1x Tris-Acetate EDTA (TAE) buffer

The 1x Tris-Acetate EDTA buffer was used in the process of gel electrophoresis. This buffer was prepared by adding 100ml of the concentrated 10x TAE buffer stock solution to 900ml of distilled water.

2.5.10.5 Preparation of 2% agarose gel

A 2% agarose gel was prepared by dissolving 4g of agarose powder in 200ml of 1x Tris-Acetate EDTA (TAE) buffer in a 500ml Erlenmeyer flask. The mixture was heated in the microwave with

frequent mixing in between until completely dissolved. The gel was left to cool down to 50°C, followed by addition of 50µl of ethidium bromide dye. The gel was mixed gently and poured onto a casting chamber and was left to solidify.

2.5.10.6 Gel electrophoresis

An electrophoresis tray was filled with 1x TAE buffer and the 2% solidified agarose gel was gently placed in it. A 1 000 base pair DNA ladder was loaded on position 1, followed by a positive and negative control and the 18 amplicons for the isolates. The gel electrophoresis was run at 110 volts for 45 minutes.

2.5.10.7 Gel visualisation

After completion of electrophoresis, the gel was removed from the electrophoresis tray. It was then placed on the SYN GENE BIO IMAGING ultra violet system for DNA visualising. The sizes of DNA fragments were identified through size using the 1 000 base pair ladder as a DNA molecular size standard. The *scp*B gene is associated with an amplicon size of 255bp.

2.5.11 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing for CAMP positive isolates was performed using both the Kirby-Bauer disk diffusion method described in the Clinical Laboratory Standard Institute (CLSI) guidelines 2018 and the Vitek 2 system. Before sensitivity testing, glycerol frozen GBS isolates were thawed and inoculated on blood agar and streaked for single colonies, followed by incubation at 37°C for 24 hours.

Penicillin, ampicillin, ceftriaxone, erythromycin, clindamycin, vancomycin, tetracycline, linezolid & chloramphenicol were the only antibiotics reported in this study, as they are recommended for testing against streptococcus (CLSI, 2018). Inducible clindamycin resistance was determined using Vitek 2 and using the disc diffusion method as described before.

DensiCheck PLUS turbidity meter

This is a McFarland turbidity meter which was used in measurement of turbidity of bacterial suspensions for manual and Vitek 2 sensitivity testing (bioMerieux, Midrand, South Africa). This turbidity meter was initially controlled prior to use, by using controls of known turbidity i.e. 0.00, 0.50, 0.20 and 0.30 McFarland. The turbidity meter was then used for preparation of bacterial suspensions after controls were deemed acceptable.

Kirby-Bauer disc diffusion method

A direct colony suspension method was used to prepare bacterial inoculums equivalent to a 0.5 McFarland standard, measured using a DensiCheck turbidity meter (CLSI, 2018). A sterile cotton-tipped swab was dipped into the test suspension, pressed gently against the tube and inoculated onto the entire surface of the blood agar. Using a sterile needle, antibiotic discs (maximum 6) were placed onto the surface of the agar with sufficient spacing.

The plates were incubated at 37°C for 24 hours within 15 minutes of disc placement. After incubation, antimicrobial sensitivity was determined and zones were measured using a pair of callipers and where graded as sensitive, intermediate sensitive or resistant using the CLSI guidelines (CLSI, 2018).

Vitek 2 sensitivities method

Preparation of suspensions for Vitek 2 sensitivities

A direct colony suspension method was used to prepare bacterial inoculums equivalent to 0.5 McFarland, measured using a DensiCheck turbidity meter (CLSI, 2018). This was done by emulsifying sufficient growth of the test isolate in 3 ml of sterile saline in a Vitek tube using a sterile cotton-tipped swab.

Gram positive Vitek 2 sensitivity cards were then programmed and loaded onto the Vitek with the bacterial suspensions for sensitivity testing. After completion, the organism was selected as *S. agalactiae* and the MIC values were printed from the Vitek 2 and were reviewed.

2.5.12 Gene based resistance testing using multiplex PCR

Multiplex PCR was performed to determine the genetic basis of resistance in the GBS isolates against tetracycline only, to which all isolates were phenotypically resistant. In this method, the elongation factor proteins *tet*(M) and *tet*(O) were amplified as target genes through multiplex PCR as described before (Zeng *et al*., 2006).

2.5.12.1 Preparation of PCR primers

Multiplex PCR for gene based resistance was performed using primers illustrated in Table 2.3. The primer's stock solution of 100µM and working solution of 10µM were prepared as per the manufacturer's instructions (Inqaba Biotechnical Industries, Pretoria, South Africa).

Table 2.3: Primers used for multiplex PCR for gene based resistance testing

Primer	Target	Primer sequence 5' – 3'	Size
*tet*mS – F	*tet*(M)	GTCTTGCATATATACGCCTTTATAGTGGAGTACTACATTTACGAG	374bp
*tet*mA – R	*tet*(M)	CCACGTAATATCGTAGAAGCGGATCACTATCTGAG	
*tet*oS – F	*tet*(O)	CGTATATATAGCGGAACATTGCATTTGAGGG	548bp
*tet*oA – R	*tet*(O)	CGGCTCTATGGACAACCCGACAGAAG	

Adapted from Poyart *et al*., 2003; Zeng *et al*., 2006

2.5.12.2 Multiplex PCR for gene based resistance testing

A mixture of PCR components was prepared by transferring nuclease free water, PCR master mix, forward and reverse primers as illustrated in Table 2.4.

Table 2.4: Preparation of multiplex PCR components for gene based resistance testing

PCR component	Volume
PCR master mix with colour	240µl
Nuclease free water	210µl
*tet*mS forward primer	20µl
*tet*mA reverse primer	20µl
*tet*oS forward primer	20µl
*tet*oA reverse primer	20µl
Total volume	530µl

The solution was mixed gently and dispensed in 26.5µl aliquots into 20 PCR tubes labelled from 1 – 18, with a positive and negative control included. Exactly 5µl of the already extracted DNA, positive control and negative control (nuclease free water) was added to each respective 0.2ml PCR tube, followed by gentle mixing. The PCR tubes were loaded in the thermal cycler (BioRad, Johannesburg, South Africa) and conditions illustrated in the Table 2.5 were applied.

Table 2.5: Multiplex PCR conditions for gene based resistance testing

PCR cycles	Phase	Temperature	Time
1x	Initial denaturation	94 °C	4 minutes
	Denaturation	93 °C	1 minute
35x	Annealing	57.6 °C	1 minute
	Elongation	72 °C	1 minute
1x	Prolonged elongation	72 °C	7 minutes
Dwell	Hold	4 °C	Infinity

After completion of the PCR cycles, the PCR amplicons were run on a 2% agarose gel electrophoresis at 110 volts for 45 minutes and the gel was visualised using the SYN GENE BIO IMAGING ultra violet system for DNA visualising as described before. The DNA fragments were identified through size using the 1 000 base pair ladder as a DNA molecular size standard.

2.5.13 Polysaccharide capsular typing for GBS isolates

Singleplex PCR was performed to determine the polysaccharide capsular types. All solates were typed into the common capsular types, i.e. Ia, Ib, II, III, IV, V, VI, VII, VIII and IX using specific primers described before (Poyart *et al*., 2007).

2.5.14 Preparation of PCR primers for capsular typing

Prior to capsular typing, the primer's stock solution of 100µM and working solution of 10µM were prepared as per the manufacturer's instructions (Inqaba Biotechnical Industries, Pretoria, South Africa). Table 2.6 illustrates the primers used in polysaccharide capsular typing.

Table 2.6: Primers used in singleplex PCR for polysaccharide capsular typing

Primer	Target	Primer sequence	Size bp
Ia-F	*cps1aH*	5' – GGTCAGACTGGATTAATGGTATGC – 3'	521 and
Ia-R	*cps1Ah*	5' – GTAGAAATAGCCTATATACGTTGAATGC – 3'	1,826 bp
Ib-F	*cps1bJ*	5' – TAAACGAGAATGGAATATCACAAACC – 3'	770 bp
Ib-R	*cpsIbK*	5' – GAATTAACTTCAATCCCTAAACAATATCG – 3'	
II-F	*cps2K*	5' – GCTTCAGTAAGTATTGTAAGACGATAG – 3'	397 bp
II-R	*cps2K*	5' – TTCTCTAGGAAATCAAATAATTCTATAGGG – 3'	
III-F[a]	*cps1a/2/3I*	5' – TCCGTACTACAACAGACTCATCC – 3'	1,826 bp
III-R[a]	*cps1a/2/3J*	5' – AGTAACCGTCCATACATTCTATAAGC – 3'	
IV-F	*cps4N*	5' – GGTGGTAATCCTAAGAGTGAACTGT – 3'	578 bp
IV-R	*cps4N*	5' – CCTCCCCAATTTCGTCCATAATGGT – 3'	
V-F	*cps5O*	5' – GAGGCCAATCAGTTGCACGTAA – 3'	701 bp
V-R	*cps5O*	5' – AACCTTCTCCTTCACACTAATCCT – 3'	
VI-F	*cps6I*	5' – GGACTTGAGATGGCAGAAGGTGAA – 3'	487 bp
VI-R	*cps6I*	5' – CTGTCGGACTATCCTGATGAATCTC – 3'	
VII-F	*cps7M*	5' – CCTGGAGAGAACAATGTCCAGAT – 3'	371 bp
VII-R	*cps7M*	5' – GCTGGTCGTGATTTCTACACA – 3'	
VIII-F	*cps8J*	5' – AGGTCAACCACTATATAGCGA – 3'	282 bp
VIII-R	*cps8J*	5' – TCTTCAAATTCCGCTGACTT – 3'	

Adapted from Poyart *et al*., 2007

2.5.14.1 Singleplex PCR for polysaccharide capsular typing

Singleplex PCR for capsular typing was carried out using primers in Table 3.6. PCR components were prepared by adding each primer pair to an eppendorf tube containing 240µl of PCR master mix and 210µl of nuclease free water, forming a total volume of 490µl. The solutions were mixed gently and dispensed in 24.5µl aliquots into 0.2ml PCR tubes, with a positive and negative control included. Exactly 5µl of the already extracted DNA, positive control and negative control (nuclease free water) was added to respective PCR tubes.

The PCR tubes were loaded in the thermal cycler in different categories and conditions shown in Table 2.7 were applied, and the annealing temperature was changed respectively from category 1 for capsular type Ia – III, category 2 for type IV – VII and category 3 for type VIII and IX.

Table 2.7: Singleplex PCR conditions for polysaccharide capsular typing

PCR cycles	Phase	Temperature	Time
1x	Initial denaturation	94 °C	4 minutes
	Denaturation	93 °C	1 minute
	Annealing category 1	58 °C	1 minute
35x	Annealing category 2	59 °C	1 minute
	Annealing category 3	56 °C	1 minute
	Elongation	72 °C	1 minute
1x	Prolonged elongation	72 °C	7 minutes
Dwell	Hold	4 °C	Infinity

After completion of the PCR cycles, the amplicons were run on a 2% agarose gel electrophoresis at 110 volts for 45 minutes and the gel was visualised using the SYN GENE BIO IMAGING ultra violet system for DNA visualising as described before. The DNA fragments were then identified through size using a 1 000bp ladder as a DNA molecular size standard.

2.6 Methods of data analyses

Data was analysed using the Statistical Package for Social Sciences (SPSS) version 22, in which the Pearson's Chi square was used. Descriptive statistics were used to summarise the frequencies and to compute frequency and cross tables. A *P* value less than 0.05 was considered significant.

2.6.1 Ethical considerations

Authority to conduct this study was granted by the Ministry of Health and Social Services and Namibia University of Science and Technology's department of Health Sciences' ethical committees. A written consent was sought from each participant in order to show willingness to participate in this study.

Confidentiality was maintained as no participant names were used in this study, Participants' data was kept on a password protected device and questionnaires were destroyed after capturing. Registered nurses were trained on how to collect the specimens. A lower vaginal and rectal swab was collected from each participant, which require a less invasive collection procedure, therefore minimal risk was posed to the unborn babies and pregnant women who participated in this study.

CHAPTER 3: RESULTS

3. RESULTS

3.1 Prevalence of Group B streptococcus

A total of 210 pregnant women at 35+ weeks of gestation were screened in this study between May and September 2018, of which 98 were screened at Onandjokwe, 93 at Eenhana and 19 at Okongo state hospitals. Out of the 210 screened pregnant women, 12 (5.7%) were colonised by GBS. The prevalence of GBS colonisation was therefore 5.7%, as shown in Table 3.1.

Table 3.1 Prevalence of GBS colonisation in pregnant women at 35+ weeks of gestation screened at Onandjokwe, Eenhana and Okongo state hospitals

		Frequency	Percentage
Culture results	Negative	199	94.8%
	Positive	12	5.7%
	Total	210	100%
			n = 210

3.2 Recovery sites for Group B streptococcus screening

Out of the total 210 pregnant women screened, 10 (4.8%) tested positive with vaginal swabs and 200 (95.2%) tested negative, while with rectal swabs 5 (2.3%) tested positive and 205 (97.6%) tested negative. Only 3 (1.43%) out of the total 210 tested positive with both rectal and vaginal swabs.

Out of the total 205 women who tested negative by rectal screening, 7 (3.4%) tested positive by vaginal screening. Out of the 200 women that tested negative by vaginal screening, only 2 (1%) tested positive by rectal screening.

A total of 15 GBS isolates were isolated in this study, i.e. 10 isolated using lower vaginal swabs and 5 using rectal swabs. This is shown in Table 3.2.

Table 3.2: Comparison of overall GBS isolation from vaginal and rectal swabs by both direct BCNA and Todd-Hewitt broth culture

		Lower vaginal swabs isolation		Total
		Negative	Positive	
Rectal swabs isolation	Negative	198	7	205
	Positive	2	3	5
	Total	200	10	210

P value = 0.001 (n = 210)

3.3 Culture media for isolation of Group B streptococcus

All 210 lower vaginal and rectal swabs were cultured directly onto BCNA agar and in Todd-Hewitt broth, followed by subculture onto BCNA agar. Out of the 210 lower vaginal swabs cultured, 4 (1.9%) tested positive by direct BCNA culture while 206 (98.1%) tested negative.

Out of the 206 that tested negative with direct BCNA culture, 6 (2.9%) tested positive with Todd-Hewitt broth-BCNA culture. However, out of the 4 (1.9%) that were positive by direct BCNA culture, 2 (50%) were negative by Todd-Hewitt broth-BCNA culture. This is shown in Table 3.3.

Out of the 210 rectal swabs cultured, 2 (0.95%) tested positive and 208 (99.0%) negative by direct BCNA culture. On the contrary, 3 (1.4%) tested positive and 207 (98.6%) negative by Todd-Hewitt broth-BCNA culture. This is illustrated in Table 4.3.

Table 3.3: Comparison of GBS isolation from vaginal and rectal swabs using direct BCNA culture and Todd-Hewitt-BCNA culture

			Direct BCNA culture		Total	P value
			Negative	Positive		
Vaginal swabs	Todd-Hewitt-BCNA culture	Negative	200	2	202	0.001
		Positive	6	2	8	
		Total	206	4	210	
Rectal swabs	Todd-Hewitt-BCNA culture	Negative	205	2	207	0.864
		Positive	3	0	3	
		Total	208	2	210	

3.4 Risk factors for GBS colonisation in pregnant women

The influence of different socio-demographic factors, age and obstetric factors on GBS colonisation in pregnant women was assessed in this study. The age of pregnant women

screened in this study ranged from 16 – 49 years with mean age of 29 years and standard deviation of ± 7.

Out of the total 210 pregnant women, 174 were living in rural, 9 in semi-urban and 27 in urban areas. One hundred and sixty women (160) were not married, while 50 were married. Those with qualifications below matric were 156, those with matric qualifications were 51 and those with tertiary qualifications were only 3. Unemployed women were 168 and the employed ones were 42.

Out of the twelve colonised women, 75.0% was women aged between 20 – 39 years and 25.0% was women aged 40 years and above (*P* value 0.209). Majority of colonised women were rural based women (83.3%) than urban living women (16.7%) (*P* value 0.708). Unmarried women colonised by GBS were 75.0% and 25.0% of colonised women were married (*P* value 0.921). These differences were not statistically significant, as indicated by the *P* values > 0.05.

Majority of colonised women were those with educational level below matric (58.3%) than those with matric qualifications (41.7%) (*P* value 0.333). The same scenario was found in terms of employment status, whereby 66.7% of unemployed women were colonised and only 33.3% of colonised women were employed (*P* value 0.234).

Majority of GBS colonised were unmarried, unemployed and rural based women aged between 20 – 39 years, with educational level below matric. Table 3.4 illustrates the rates of GBS colonisation based on various socio-demographic factors.

Table 3.4: Association between socio-demographic factors and GBS colonisation

Characteristic	Category	GBs non-colonised frequency (n = 198)	GBS colonised frequency (n = 12)	*P* value
Age in years	Below 20	12 (6.1%)	0 (0.0%)	0.209
	20 – 39	166 (83.8%)	9 (75.0%)	
	40 and above	20 (10.1%)	3 (25.0%)	
Habitat	Rural	164 (82.8%)	10 (83.3%)	0.708
	Semi-urban	9 (4.5%)	0 (0.0%)	
	Urban	25 (12.6%)	2 (16.7%)	
Marital status	Not married	151 (76.3%)	9 (75.0%)	0.921
	Married	47 (23.7%)	3 (25.0%)	
Educational level	Below matric	149 (75.3%)	7 (58.3%)	0.333
	Matric	46 (23.2%)	5 (41.7%)	
	Tertiary	3 (1.5%)	0 (0.0%)	
Employment status	Unemployed	160 (80.8%)	8 (66.7%)	0.234
	Employed	38 (19.2%)	4 (33.3%)	

The influence of different maternal obstetric factors on GBS colonisation was also analysed. This includes parity, history of still births and history of miscarriages. Among all GBS colonised women, women of with three or more successful pregnancies were 50.0%, while those with successful pregnancies between one and two were 33.0% and those without any previous pregnancy were 16.7% (*P* value 0.659). These differences were not statistically significant.

Carriage of GBS was only shown in women without previous history of still births (*P* value 0.613). GBS colonised women without any history of miscarriages were 83.3% while those with a history of 1 – 2 miscarriages were 16.7%, however these differences were not statistically significant (*P* value 0.471).

Table 3.5: Association between obstetric factors and GBS colonisation

Characteristic	Category	GBS non-colonised frequency (n = 198)	GBS colonised frequency (n = 12)	*P* value
Parity	0	43 (21.7 %)	2 (16.7 %)	0.659
	1 – 2	82 (41.4 %)	4 (33.3 %)	
	≥3	73 (36.9 %)	6 (50.0 %)	
Still births	0	183 (92.4 %)	12 (100 %)	0.613
	1 – 2	13 (6.6 %)	0 (0.0 %)	
	≥3	2 (1.0 %)	0 (0.0 %)	
Miscarriages	0	178 (89.9 %)	10 (83.3 %)	0.471
	1 – 2	20 (10.1 %)	2 (16.7 %)	
	≥3	0 (0.0 %)	0 (0.0 %)	

3.5 Presumptive identification and confirmation of Group B streptococcus

The culture of rectal and lower vaginal swabs by both direct BCNA agar and Todd-Hewitt-BCNA agar has yielded a total of 15 CAMP positive presumptive GBS isolates. These isolates were confirmed as *S. agalactiae* using real time PCR, in which the target gene *scp*B for C5a peptidase gene was amplified.

Following gel electrophoresis, DNA amplicons approximately 255 base pairs on the gel were identified as *S. agalactiae* as previously described by Dmitriev *et al*., 2004. No sequencing was performed in this study. Figure 3.1 represents a sample of amplicons of the *scp*B gene for *Streptococcus agalactiae* visualised under ultra-violet light.

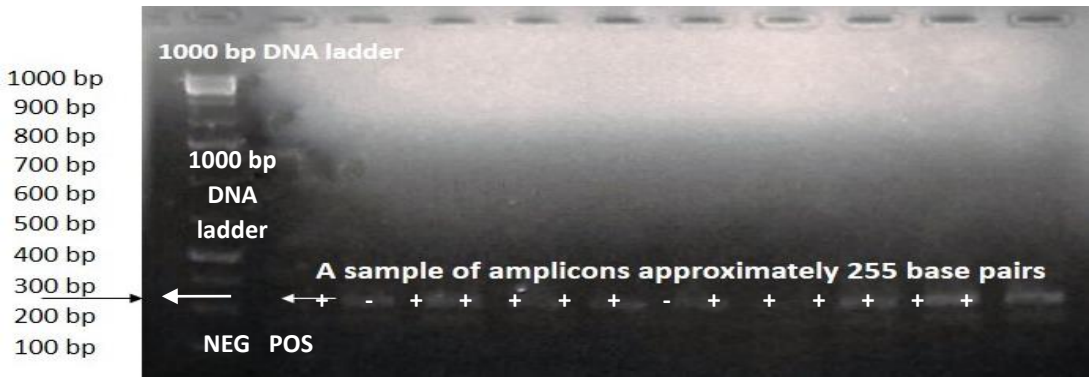

Figure 3.1: A sample of amplicons for *scp*B visualised under the SYN GENE BIO IMAGING ultra violet system

3.6 Antimicrobial susceptibility patterns

The total number of antibiotics evaluated in this study was eight, which were selected using the Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2018). All 15 GBS isolates obtained from this study were tested for antimicrobial susceptibility, and all had shown 100% susceptibility to penicillin, ampicillin, ceftriaxone, clindamycin, erythromycin, vancomycin, linezolid and chloramphenicol.

All isolates were resistant to tetracycline (100%). Neither intermediary sensitivity, nor inducible clindamycin resistance was reported in this study. The frequencies of antimicrobial susceptibility patterns are illustrated in Table 3.6.

Table 3.6: Frequency of antimicrobial susceptibility patterns of GBS isolates (n = 18)

Antimicrobial agent	Percentage of GBS antibiotics sensitive, intermediate and resistant					
	Sensitive (S)		Intermediate (I)		Resistant (R)	
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage
Penicillin	15	100%	0	0%	0	0%
Ampicillin	15	100%	0	0%	0	0%
Ceftriaxone	15	100%	0	0%	0	0%
Erythromycin	15	100%	0	0%	0	0%
Clindamycin	15	100%	0	0%	0	0%
Vancomycin	15	100%	0	0%	0	0%
Tetracycline	0	0%	0	0%	18	100%
Linezolid	15	100%	0	0%	0	0%
Chloramphenicol	15	100%	0	0%	0	0%

Ceftriaxone was performed using Kirby-Bauer disc diffusion method only, whereas the rest of the antibiotics were performed using Vitek 2, except for isolate 16 and 18 which were tested manually against all antibiotics.

Consistent minimum inhibitory concentrations (MICs) were observed in most antibiotics. These include ampicillin (≤0.25µg/L), penicillin (≤0.12µg/L), clindamycin (≤0.25µg/L), chloramphenicol (≤10µg/L) and tetracycline (≥16µg/L). The highest MIC value reported in erythromycin was 0.5µg/L and the lowest was ≤0.25µg/, which were all considered sensitive.

For vancomycin, the highest MIC value reported was 4µg/L and the lowest was ≤ 0.5µg/L, which were all considered sensitive. Linezolid was reported sensitive with MICs ranging from 1µg/L to 2µg/L, which were all considered sensitive. The smallest inhibitory zone reported for ceftriaxone was 25mm and the biggest was 33mm which were all considered as sensitive.

3.7 Gene based resistance results

Previously described specific primers displayed in Table 3.3 where used in determining the genetic resistance basis of *S. agalactiae* isolates obtained in this study as described previously (Zeng *et al*., 2006). Gene based resistance multiplex PCR was carried out on 15 GBS isolates including a positive control, to determine the presence of resistance genes against tetracycline.

None of the isolates possessed *tet*(O) resistance gene, regardless of being phenotypically resistant to tetracycline. Amplicons for *tet*(M) have 347 base pairs and for *tet*(O) have 548 base pairs (Poyart *et al*., 2003). The results are illustrated in Table 3.7 and Figure 3.2.

Table 3.7: Frequency of resistance genes detected in tetracycline resistant GBS isolates

Resistance Gene		Frequency	Percentage
tet(M)	Present	16	88.9 %
	Absent	2	11.1 %
	Total	18	100 %
tet(O)	Present	0	0 %
	Absent	18	100 %
	Total	18	100 %
			(n = 18)

Figure 3.2: A sample of amplicons for *tet*(M) visualised under the SYN GENE BIO IMAGING ultra violet system

3.8 Polysaccharide capsular typing results

Polysaccharide capsular typing was performed on 18 GBS isolates using singleplex PCR as described before (Poyart *et al*., 2007). Following the amplification process, nine isolates presented with amplicons of 521 base pairs and one with an amplicon sized 770 base pairs. An amplicon of 521 base pairs is associated with capsular polysaccharide Ia whereas 770 base pairs is associated with capsular type Ib.

Polysaccharide capsular type Ia was found in 9 (50%) of isolates and Ib was found in 1 (5.5%) of the total isolates. The remaining eight (44.4%) isolates could not be classified using PCR. The frequencies of GBS capsular types are illustrated in Table 3.8 and the amplicons for capsular type Ia and Ib are shown in Figure 3.3.

Table 3.8: Frequency polysaccharide capsular types of GBS isolated from pregnant women

Capsular type	Frequency	Percent (%)
Ia	9	50.0 %
Ib	1	5.5 %
Non-typeable	8	44.4 %
Total	10	55.5 %

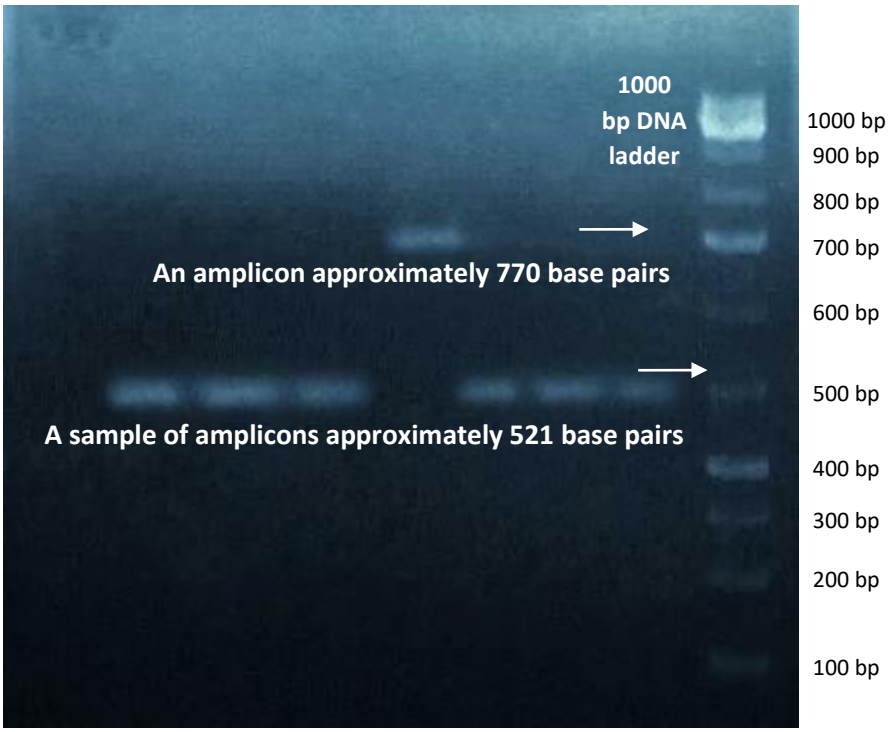

Figure 3.3: A sample of amplicons for capsular type Ia (521 bp) and Ib (770 bp) visualised under the SYN GENE BIO IMAGING ultra violet system

CHAPTER 4: DISCUSSION AND CONCLUSION

4.1 Discussion

4.1.1 Prevalence of Group B streptococcus

The current study aimed at determining the prevalence of Group B streptococcus colonisation in pregnant women at 35 weeks of gestation and above, from hospitals in Ohangwena and Oshikoto regions of Namibia, their antimicrobial susceptibility patterns and gene based resistance as well as their polysaccharide capsular types. The prevalence of GBS colonisation was found to be 5.7% in pregnant women at 35 weeks of gestation and above.

The prevalence found in this study was lower compared to what was reported in a similar study conducted in Windhoek, Namibia in 2016, whereby the prevalence of GBS colonisation was reported at 13.6% 2016 (Engelbrecht *et al*., 2016). Different recruitment centres were included in the current study, which comprise of a different population from the Windhoek study.

This finding is different from the 31.6% rate found in Zimbabwe in 2000 (Moyo *et al*., 2000), 30.9% in South Africa in 2012 (Chukwu *et al*., 2015; Monyama *et al*., 2016) and 21.2% in Malawi in 2010 (Gray *et al*., 2011).

Our finding is however similar to what was found in a Mozambican study, whereby a colonisation rate of 1.8% was obtained (de Steenwinkel *et al*., 2008). However, in the Mozambican study swabs were inoculated in Todd-Hewitt broth with only gentamycin (8µg/ml of agar) and the broth was sub-cultured onto human blood agar.

The use of gentamycin alone without nalidixic acid can cause overgrowth of enterobacteriaceae organisms over GBS, hence reducing the growth of GBS. Furthermore, human blood used in preparation of blood agar might have contained inhibitory substances against the growth of GBS (de Steenwinkel *et al*., 2008).

4.1.2 Recovery sites for Group B streptococcus screening

Our study has shown that 58% of colonised women were colonised vaginally, 17% rectally and 25% both rectally and vaginally. In the Windhoek study, 46.25% of the colonised women were colonised in both the lower vagina and rectum, 36.25% in the lower vagina and 17.5% in the rectum only (Engelbrecht *et al*., 2016). Although similar isolation medium was used, a lower isolation rate was obtained from our study. This may be because the Windhoek study used an additional differential medium for GBS (Granada agar), and more isolates were obtained compared to the current study.

In the current study, 10 (4.8%) out of the 210 pregnant women screened were positive by lower vaginal screening and only 5 (2.3%) were positive by rectal screening. This implies that the recovery of GBS from the lower vagina was relatively higher than recovery from the rectum. It was observed that 7 (3.4%) out of 205 women with negative rectal screening were positive by vaginal screening.

4.1.3. Culture media for isolation of Group B streptococcus

In the current study, 4 (1.9%) out of the total women screened in the lower vagina tested positive by direct BCNA culture while 206 (98.1%) tested negative. However, it was observed that among the 206 that tested negative with direct BCNA culture, 6 (2.9%) tested positive with Todd-Hewitt broth-BCNA culture. This was a significant statistical finding as confirmed with a *P* value of 0.001. This suggests that the involvement of enrichment broth supplemented with antibiotics can possibly increase the isolation of Group B streptococcus and increase the recovery of GBS even when present in lower quantities. When only traditional culture (direct agar plating) is used without additional selective enrichment broth, about 50% of women who are GBS colonised might yield false negative culture results (CDC, 2010).

4.1.4. Presumptive identification and confirmation of Group B streptococcus

Presumptive identification of GBS isolates was done using the CAMP test, and all isolates were confirmed as *S. agalactiae* by real time PCR, in which the target gene *scp*B was amplified. All CAMP positive isolates have shown presence of an amplicon of size 255 base pairs on the electrophoresis gel, confirming the organism as GBS. The results of the two tests have shown 100% correlation.

4.1.5. Risk factors

Generally, GBS colonisation did not appear to be influenced by age, geographic location, marital status, educational level and employment status. This study revealed that 75.0% of colonised women were aged between 20 – 39 years and 25.0% were aged 40 years and above. There was however no statistical significant association between GBS colonisation and age (*P* value 0.209). A similar study conducted at Dr George Mukhari hospital, South Africa found majority of GBS colonised women to be aged between 25 and 29 years, which was also not statistically significant (*P* value 0.07) (Monyama *et al*., 2016). There was a significant association between maternal age and GBS colonisation in pregnant women screened at the maternity and children hospital in Makkah, Saudi Arabia (*P* value 0.002) (Khan *et al*., 2015).

In the current study, 83.3% of colonised women were rural based and 16.7% were urban based. This difference was however not statistically significant (*P* value 0.708). In a similar study conducted in Dr George Mukhari hospital, South Africa, more of the colonised women were dwelling in the urban areas (Monyama *et al*., 2016). This finding was however also not statistically significant (*P* value 0.08). This difference may be attributed to differences in the ethnic groups of inhabitants of the two locations.

It was observed in this study that majority of GBS colonised women were unmarried women 75.0%, compared to married women 25.0%. There was however no significant statistical association between GBS colonisation and marital status (*P* value 0.921). This agrees with the study conducted at Dr George Mukhari hospital, South Africa, where majority of colonised women were single, divorced or widowed women followed by cohabiting women (*P* value 0.56) (Monyama *et al*., 2016). In another study conducted in Windhoek, more single women were colonised compared to married women (*P* value 0.315) (Engelbrecht *et al*., 2016). Both findings were not statistically significant.

Furthermore, GBS was more prevalent in women who had no formal secondary education (58.3%), with a decrease in colonisation observed as educational level increases, although this was statistically insignificant (*P* value 0.333). A slight decrease in GBS colonisation with increase in educational level was also observed in the study conducted in Windhoek, although this was also statistically insignificant (*P* value 0.729). On the contrary, educational level was found to have a significant influence on GBS colonisation in pregnant women in the South

African study (Dr George Mukhari hospital), whereby women with tertiary level of education were found to have lower colonisation compared to women with and without secondary education (*P* value < 0.0001) (Monyama *et al*., 2016).

Unemployed women were largely colonised by GBS than employed women as discovered in our study, with percentages of colonisation being 66.7% and 33.3% respectively. This was however not statistically significant (*P* value 0.234). On the contrary, a slightly large number of GBS colonised women was found to be employed women as revealed in the study conducted at Dr George Mukhari hospital (Monyama *et al*., 2016) and in Windhoek (Engelbrecht *et al*., 2016). The relationship between employment and GBS colonisation was however not statistically significant in the Windhoek study (*P* value 0.125) (Engelbrecht *et al*., 2016), while in the South African study it was statistically significant (*P* value 0.003) (Monyama *et al*., 2016).

Group B streptococcus colonisation did not appear to be linked to obstetric factors such as history of miscarriages and still births, as women with no history of still births and miscarriages were largely colonised (100% and 83.3% respectively). These findings did not have any statistical significance (*P* value 0.613 and 0.471 respectively). However, GBS colonisation had shown to increase with parity, 50.0% in women with parity exceeding two, 33.3% in women with one or two previous deliveries and 16.7% in women without previous deliveries. This was however not statistically significant (*P*. value 0.659). In the Saudi Arabian study conducted at Makkah, a significant statistical association was found between parity and GBS colonisation (Khan *et al*., 2015).

Similarly, GBS colonisation did not appear to be linked to history of miscarriages and still births in the South African study, as the carriage rate was greater in women who had no history of miscarriages and still births compared to the opposite. These were equally not statistically significant findings (*P* value 0.33 and 0.24 respectively) (Monyama *et al*., 2016). In contrast to our findings, women with multiple deliveries exceeding two were less affected by GBS compared to women who had one to two deliveries and those with zero parity. This still did not have any statistical significance (*P* value 0.24) (Monyama *et al*., 2016).

4.1.6. Antimicrobial susceptibility patterns

In this study, GBS has shown sensitivity to penicillin, ampicillin, ceftriaxone, vancomycin, chloramphenicol and linezolid by 100%. This correlates with the findings of the study done in Windhoek, where 100% susceptibility was reported to penicillin, ampicillin, ceftriaxone, vancomycin and linezolid. All isolates were found to be sensitive to clindamycin and erythromycin by 100%. This does not correlate with findings of the Windhoek study, whereby 88.9% sensitivity and 11.1% resistance was reported for erythromycin, while for clindamycin 72.6% sensitivity, 18.8% intermediate sensitivity and 8.5% resistance was reported (Engelbrecht *et al*., 2016).

Since only 15 GBS isolates were obtained from the current study, 100% susceptibility to clindamycin and erythromycin might not be a genuine and true reflection of the antimicrobial susceptibility patterns of GBS in the two regions, as a bigger number of isolates might have reflected a different picture. Furthermore, our study has revealed a 100% resistance to tetracycline, while the previous study has revealed 94.9% resistance to tetracycline and 5.1% susceptibility thereof. This differences may be attributable to the differences in the locations of the two studies.

The findings of our study are in correlation with another study conducted in Ayder referral hospital and Mekelle health centre, Mekelle, Northern Ethiopia, whereby GBS was reportedly 100 % susceptible to penicillin, ampicillin, erythromycin, and vancomycin (Alemseged *et al*., 2015). However, in the same study, intermediate GBS susceptibility was reported to chloramphenicol by 42.1% and ceftriaxone by 26.3%, which opposes the findings of our study (Alemseged *et al*., 2015).

On the contrary, our study findings are different from the findings of a similar study conducted in Ghandi memorial and Tikur Anbessa, Addis Ababa, Ethiopia, where GBS was found to be 55% susceptible to penicillin, 91% sensitive to ampicillin and all isolates except one were susceptible to erythromycin (Woldu *et al*., 2014). Increased resistance to penicillin in this area was attributable to a wide non-prescription use of penicillin due to weak drug control mechanism (Woldu *et al*., 2014).

Another study conducted in Gabon, Central Africa to determine capsular type distribution and antimicrobial susceptibility of GBS in pregnant women found that all GBS isolates were

susceptible to clindamycin, linezolid, vancomycin and benzylpenicilin which correlates with the findings of our study (Belard *et al*., 2015). Fourteen isolates were however found intermediary susceptible to erythromycin, which contrasts the findings of our study. However, no inducible clindamycin resistance was found in the Gabon study, which draws a parallel between the two studies (Belard *et al*., 2015).

In the neighbouring South Africa, a study conducted to assess the antimicrobial resistance of *S. agalactiae* isolated from pregnant women in Garankuwa found that all strains were 100 % susceptible to penicillin, ampicillin and vancomycin, which is similarly demonstrated in our study (Bolukaoto *et al*., 2015). The same study however found some strains to be resistant to erythromycin, clindamycin, tetracycline and chloramphenicol by 21.1%, 17.2%, 94.5% and 24.9% respectively, which distinguishes the two study findings (Bolukaoto *et al*., 2015).

In a comparable study conducted in Soweto, South Africa to determine the risk of GBS sepsis in infants exposed to HIV, all isolates were penicillin sensitive, and macrolide resistance was observed in 5.5% (Cutland *et al*., 2015).

Generally, GBS has shown uniform sensitivity to penicillin, ampicillin, vancomycin, ceftriaxone and linezolid across many settings. Resistance to tetracycline was reported at 100 % in our study compared to many previous studies, even though they also reported quite high percentages of resistance to tetracycline. Resistance to erythromycin and clindamycin was however not reported in our study, as opposed to other studies.

These differences may be attributed to variations in capsular types common in different locations, hence the differences in their antimicrobial susceptibility patterns. Also, fewer GBS isolates were obtained in the current study as compared to other studies, hence the differences in percentages of antimicrobial susceptibility.

4.1.7. Gene based resistance of *S. agalactiae*

Our study revealed 100% resistance to tetracycline, as all isolates were resistant. Of these 18 isolates, 16 (88.9%) presented with band sizes of 347 base pairs on the electrophoresis gel, following multiplex PCR amplification in which primers for *tet*(M) and *tet*(O) were used as described before. Two isolates however did not present with any bands on the electrophoresis gel, regardless of being resistant to tetracycline phenotypically.

An amplicon size of 347 base pairs is associated with *tet*(M), a known gene that codes for resistance against tetracycline (Zeng *et al*., 2006). None of the isolates phenotypically resistant to tetracycline presented with an amplicon size of 548 base pairs, which is associated with *tet*(O) gene, which also confers resistance against tetracycline (Zeng *et al*., 2006).

The protein coded for by *tet*(M) is an elongation factor which interacts with the ribosome and dislodges tetracycline from its target site. This interaction alters the ribosomal conformation, thus preventing rebinding of tetracycline (Munita & Arias, 2016). This reduces the target site's affinity for the antibiotic molecule, thus causing resistance. The absence of *tet*(M) and *tet*(O) in some GBS isolates may imply that such strains possess different mechanisms of resistance against tetracycline other than alteration of the antibiotic binding site. It might also imply minimal expression of the gene.

A related study which was conducted in Windhoek has found *tet*(M) in 114/117 GBS isolates and of these, 111 were tetracycline resistant as interpreted by the vitek (Engelbrecht *et al*., 2016). Based on that study, six of the isolates presenting with a band size of 347 base pairs did not appear to be phenotypically resistant to tetracycline (Engelbrecht *et al*., 2016). This does not concur with our findings, as all isolates presenting with *tet*(M) were phenotypically resistant to tetracycline in our study. Only tetracycline resistance genes were inspected in our study, as all isolates from this study have only shown phenotypical resistance to tetracycline.

4.1.8. Polysaccharide capsular types

Our study found capsular type Ia to be common among the pregnant women screened. A similar study conducted to determine the antigenic distribution of *S. agalactiae* isolates from pregnant women at Garankuwa hospital in South Africa revealed that capsular type III was the most common capsular type (29.7%), followed by Ia (25.6%), II (15.6%) IV (8.6%), V (10.9%) and Ib (8.6%) (Chukwu *et al*., 2015). This implies that the prominent capsular types associated with this population were III, Ia and II. In the current study, capsular type III was not picked up using PCR. Differences in locations of the two studies may attribute to the differences in capsular types found in the two studies.

Another South African study conducted on pregnant mothers attending prenatal clinics in Soweto concluded that capsular type III was more associated with persistent colonisation throughout the study (29%) than Ia (18%) and V (6%) (Kwatra *et al*., 2014). In a Zimbabwean

study, the following capsular types were identified as follows: Ia, Ib, II, III and V at 15.7%, 11.6%, 8.3%, 38.8% and 24.0% respectively (Mavenyengwa *et al*., 2010), which corresponds with our findings. In Australia, GBS capsular type Ia, II and V were the leading causes of invasive infection (Zhao *et al*., 2008).

4.2. CONCLUSION

In the current study, the prevalence of GBS colonisation in women who attended antenatal screening at Okongo, Eenhana and Onandjokwe state hospitals was 5.7%. Twelve women were colonised in total and of these, 25.0 % carried GBS in their lower vagina and rectum, 58.0% in the lower vagina only and 17.0% in the rectum only.

Our study findings have established that there is statistically significant association between the use of both rectal and lower vaginal swab, as well as between the use of direct BCNA and Todd-Hewitt-BCNA agar culture. This suggests that inclusion of both rectal and vaginal swabs in GBS screening can feasibly increase the chances of GBS isolation.

Our study findings have revealed that there was no significant statistical association between GBS colonisation and age, habitat, marital status, educational level and employment status. Similarly, there was also no statistically significant association between GBS colonisation and parity, history of still births and miscarriages.

Antimicrobial susceptibility was reported at 100% sensitive for ampicillin, penicillin, ceftriaxone, clindamycin, erythromycin, vancomycin, linezolid and chloramphenicol. Resistance to tetracycline was reported at 100%. However, 100 % susceptibility to erythromycin and clindamycin may be due to a smaller number of GBS isolates obtained from this study.

The tetracycline resistance gene *tet*(M) was present in 88.9% of isolates and 11.1% had none, regardless of being phenotypically resistant to tetracycline. None of the isolates possessed detectable *tet*(O) resistance gene.

Polysaccharide capsular type Ia was found in 9 (50%) of isolates and Ib was found in 1 (5.5%) of the total isolates. The remaining 8 (44.4%) isolates could not be classified using PCR.

4.3. LIMITATIONS

A representative number of pregnant women in Ohangwena and Oshikoto regions of Namibia could not be obtained, as only one hospital was studied in Oshikoto region and two hospitals in Ohangwena region due to ease of accessibility.

The prevalence of GBS colonisation obtained from this study may therefore not be a genuine and true reflection and cannot be generalized to the entire population of pregnant women in the two regions. Therefore, the findings of this study only apply to pregnant women who were screened at Onandjokwe, Okongo and Eenhana state hospitals between May and September 2018.

Due to limited participants, an equal number of participants could not be recruited at all recruitment centres. In addition, the gestational age range was extended from 35 – 37 weeks of gestation to 35 weeks of gestation and above in order to obtain the sufficient sample size.

Due to a smaller number of GBS isolates obtained in this study, 100% susceptibility to erythromycin and tetracycline may not be a genuine and true reflection of resistance patterns in the given regions.

Eight GBS isolates were untypeable using PCR. In addition, capsular type IX was not investigated in this study. For this reason, all unclassified GBS isolates will further be classified using a latex agglutination kit.

There was no positive control for resistance genes *tet*(M) and *tet*(O).

4.4. SIGNIFICANCE OF THE STUDY

To the Ministry of Health and Social services

This study will show a glance regarding the prevalence of GBS colonisation in pregnant women who attended antenatal screening at Onandjokwe, Eenhana and Okongo state hospitals. These finding may be used to see whether any interventions need to be instituted to reduce GBS colonisation in pregnant women and prevent invasive neonatal GBS infection, which can possibly reduce the rising numbers still births and miscarriages in Namibia.

To the Namibia Institute of Pathology

The findings of this study may provide useful information to the national diagnostic laboratory regarding the advantages of using enrichment medium in the screening of GBS colonisation during pregnancy, which can possibly increase the chances of isolating GBS and reduce false negative results.

Our study findings will also provide useful information regarding the advantages of performing rectovaginal GBS screening using both a rectal and lower vaginal swab, which can increase the chances of GBS isolation.

To the community

This study provides opportunity for further research the communities. This will provide a clear image of where Namibia is standing regarding GBS colonisation, and will help in determining the need of further instituting measures to curb still births and miscarriages that might result from gestational GBS colonisation.

4.5. RECOMMENDATIONS

The use of both rectal and lower vaginal swabs for the screening of GBS colonisation in pregnant women is suggested, as this can feasibly increase the rate of GBS isolation and mitigate false negative results. Moreover, a larger and more representable sample size of pregnant women need to be screened for Group B streptococcus colonisation from 35 weeks of gestation and above. This will generate more representative prevalence and resistance patterns compared to the current study.

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4. APPENDICES

APPENDIX A: DEFINITION OF KEY TERMINOLOGIES

- **Adhesin** Cell surface proteins which help bacterial cells to attach themselves to host cells and invade the cells to cause invasive diseases.
- **Ascending infection** An infection of the neonate which results from vaginal GBS colonisation spreading upward to the cervix and uterus.
- **Bacteraemia** The presence of bacteria in the blood.
- **Chorioamnionitis** Inflammation and infection of the inner and outer foetal membranes often after preterm premature rapture of membranes.
- **Early onset disease** Infection of the neonate which presents between day one and day seven of the infant's life.
- **Endometritis** Inflammation of the mucous membranes lining the uterus due to acute or chronic infection.
- **Gene based resistance** Antimicrobial resistance that occurs due to the presence of genes that code for resistance against certain antibiotics.
- **Group B streptococcus** Gram positive bacterium known as *Streptococcus agalactiae* that causes life threatening infections in infants.
- **Haematogenous infection** Transmission of bacteria from the mother to the infant through placental blood circulation.
- **Lancefield classification** A classification of streptococcus bacteria based on the presence of antigenic carbohydrates on the cell surface.
- **Late onset disease** Infection of the neonate which presents between day eight and day ninetieth (third month) of the infant's life.
- **Meningitis** Inflammation of the meninges due to infection by viruses, fungi or bacteria.
- **Pneumonia** Inflammation of the lung tissues caused by bacteria.
- **Polymerase chain reaction** A technique of molecular genetics in which a particular sequence of DNA is isolated and amplified into multiple copies.
- **Polysaccharide capsule** A slime layer containing carbohydrates which acts as an envelope covering and protecting the bacterial surface.

- **Primers** Short DNA sequences complementary to the target DNA, used in starting DNA synthesis during PCR.
- **Sepsis** Putrefactive destruction of tissues caused by disease-causing bacteria or their toxins.
- **Virulence factor** Any characteristic that enables an organism to cause diseases and resist the body's immune system.

Adapter from Oxford, 2010

APPENDIX B: CONSENT FORM

Research title: Prevalence and molecular characterisation of Group B streptococcus in pregnant women from hospitals in Oshikoto and Ohangwena regions of Namibia

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PURPOSE

The aim of this study is to investigate the prevalence, antimicrobial sensitivities and molecular characteristics of *Streptococcus agalactiae* in pregnant women from hospitals in Oshikoto and Ohangwena regions of Namibia. *Streptococcus agalactiae* is a bacterium that causes life-threatening infections in newborn infants following vaginal delivery.

PROCEDURE

1. This study will be conducted over a period of two years.
2. Participants will include pregnant women who are at 35 weeks of gestation and above.
3. Pregnant women who are on antibiotic treatment or who have taken antibiotics two weeks prior to sample collection will be excluded from the study.
4. Participants will be screened for *Streptococcus agalactiae* colonisation in the lower vagina and rectum.
5. A rectal and lower vaginal swab will be collected from each participant by a registered nurse.
6. A questionnaire will be used to collect demographic and clinical data from each participant.
7. A voluntary written consent must be obtained from each participant, for participation and review of her medical record, for this information to be eligible for use in this study.
8. A unique identification number will be allocated to each participant to protect their identity and all information will be handled with strict confidentiality as no names will be recorded.
9. All data will be stored on a protected device and hard copies will be stored under lock and key.

BENEFITS

The study will contribute valuable information that can guide policies regarding screening and treatment of *Streptococcus agalactiae* in pregnant women in rural areas in Namibia.

RISKS, STRESS OR DISCOMFORT

If at any stage of the investigation you feel uncomfortable, you are free to withdraw.

Signature …… Date: ……

APPENDIX C: GROUP B STREPTOCOCCUS STUDY QUESTIONNAIRE

PLEASE NOTE: NO PARTICIPANT NAME SHOULD BE WRITEN ON THIS QUESTIONNAIRE

PURPOSE: This questionnaire is designed to derive information related to Group B streptococcus colonisation in pregnant women attending antenatal screening at the selected health facilities.

INCLUSION CRITERIA: A participant should **ONLY** be included in this study if her current pregnancy falls at 35 weeks of gestation or above.

EXCLUSION CRITERIA: A participant **SHOULD BE EXCLUDED** if she has taken antibiotics within the last 14 days prior to date of contact. A participant whose pregnancy fall below 35 weeks of gestation **SHOULD BE EXCLUDED** from this study.

PART I: DEMOGRAPHIC INFORMATION

Unique identification number	:	
Date attended	:	
Hospital number	:	
Name of hospital attended	:	
Date of Birth	:	

PART II SOCIO-ECONOMIC CHARACTERISTICS

Please cross (x) the appropriate box with the correct answer:

Marital status	**Married**	**Single**	**Divorced**	**Windowed**	**Cohabiting**
	Below matric		**Matric**		**Tertiary**
Highest level of education reached					
	Employed		**Self-employed**		**Unemployed**
Participant's employment status					
	Professional		**Skilled**		**Semi-skilled**
Participant's occupation					
	Employed		**Self-employed**		**Unemployed**
Husband's employment status					
	Below matric		**Matric**		**Tertiary**
Husband's highest level of education reached					
	Rent-whole/alone		**Rent-sharing**		**Own**
Accommodation					
	Urban		**Semi-urban**		**Rural**
Where do you dwell most yearly?					
State the name of the place					

PART III: OBSTETRIC HISTORY

1. Did you take antibiotics taken in the last 14 days? …….
2. Indicate number of successful pregnancies (parity) …….
3. Indicate number of previous miscarriages (if any) …….
4. Indicate number of previous stillbirths (if any) …….
5. Indicate number of previous normal vaginal deliveries (if any) …….
6. Do you have any history of pain in the lower abdomen during pregnancy? If yes, was the pain treated or not? ……..

PART IV: Current pregnancy

Please complete in writing or indicate with a cross (x) where required:

Expected date of delivery (EDD) ……..

Gestational age at current visit (weeks) ……..

Any trauma in current pregnancy? If yes, specify ……..

Height (cm) …….

Weight (to the nearest kg) …….

Mid-arm circumference (cm) …….

RPR test results …….

HIV test results …….

Unique identification numbers:

- A patient's identification number should be preceded with the first 3 letters of the hospital name example: **Eenhana: EEN001** and **Onandjokwe: ONA001** and **Okongo: OKO001**.
- The number should be recorded in the patient's health passport for traceability purposes.

APPENDIX D: ETHICAL CLEARANCE BY MINISTRY OF HEALTH AND SOCIAL SERVICES

REPUBLIC OF NAMIBIA

Ministry of Health and Social Services

Private Bag 13198 | Ministerial Building | Tel: 061 – 2032150
Windhoek | Harvey Street | Fax: 061 – 222558
Namibia | Windhoek | Email: shimenghipangelwa71@gmail.com

OFFICE OF THE PERMANENT SECRETARY

Ref: 17/3/3 **EH**
Enquiries: Mr. J. Nghipangelwa

Date: 10 October 2017

Mr. Erastus L. Haimbodi
Namibia University of Science and Technology
Windhoek
Namibia

Dear Mr. Haimbodi

Re: Prevalence and Molecular Characterization of Group B Streptococcus in pregnant women from hospitals in Oshikoto and Ohangwena Regions in Namibia.

1. Reference is made to your application to conduct the above-mentioned study.

2. The proposal has been evaluated and found to have merit.

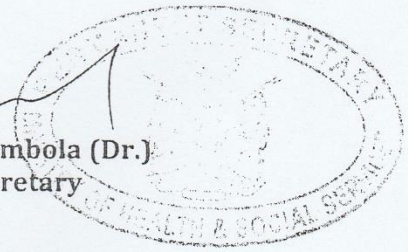
3. **Kindly be informed that permission to conduct the study has been granted under the following conditions:**

3.1 The data to be collected must only be used for academic purposes;
3.2 No other data should be collected other than the data stated in the proposal;
3.3 Stipulated ethical considerations in the protocol related to the protection of Human Subjects' should be observed and adhered to, any violation thereof will lead to termination of the study at any stage;
3.4 A quarterly report to be submitted to the Ministry's Research Unit;
3.5 Preliminary findings to be submitted upon completion of the study;

3.6 Final report to be submitted upon completion of the study;

3.7 Separate permission should be sought from the Ministry of Health and Social Services for the publication of the findings.

Yours sincerely,



Andreas Mwoombola (Dr.)
Permanent Secretary

Your Health Our Concern"

APPENDIX E: ETHICAL CLEARANCE BY THE FACULTY OF HEALTH AND APPLIED SCIENCES

NAMIBIA UNIVERSITY
OF SCIENCE AND TECHNOLOGY

13 Storch Street
Private Bag 13388
Windhoek
NAMIBIA

T: +264 61 207 9111
F: +264 61 207 2444
W: www.nust.na

FACULTY OF HEALTH AND APPLIED SCIENCES

DECISION/FEEDBACK ON RESEARCH PROPOSAL ETHICAL CLEARANCE

Dear ~~Prof/Dr/Mr~~/Ms/~~Other(s)~~: Erastus Lafimana Haimbodi

Student No (if applicable): 212113542

Research Topic:	Prevalence and molecular characterisation of Group B *Streptococcus* in pregnant women from hospitals in Oshikoto and Ohangwena Regions of Namibia
Supervisor (if applicable):	Prof SR Moyo
Co-supervisor(s): if applicable	Mr. Munyaradzi Mukesi
Qualification registered for (if applicable):	Master of Health Sciences

Re: Ethical screening application No: REC -00012/2017

The Research Ethics Screening Committee has reviewed your application for the above-mentioned research project. Based on the recommendation of the expert reviewer, the research as set out in the application is hereby:

(Indicate with an X)

Approved: i.e. may proceed with the project			
Approved provisionally: subject to compliance with recommendation(s) listed below	X		
Not approved: Not to proceed with the project until compliance with recommendation(s) listed below and resubmit ethics application for consideration			
IS MINISTRY OF HEALTH & SOCIAL SERVICES (MoHSS) APPROVAL REQUIRED?	YES:	√	NO:

It is important to note that as a researcher, you are expected to maintain ethical integrity of your research, strictly adhere to the ethical policy of NUST, and remain within the scope of your research proposal and supporting evidence as submitted to the REC. Should any aspect of your research change from the information as presented, which could have an impact or effect on any research participants/subjects/environment, you are to report this immediately to your supervisor or REC as applicable in writing. Failure to do so may result in withdrawal of approval. Kindly consult your supervisor or HoD if you need further clarification.

We wish you success in your research endeavour and are of the belief that it will have positive impact on your career as well as the development of NUST and the society in general.

Ethical issues that require compliance/ must be addressed		
No.	Ethical issues	Comment/recommendation
1.	Research activities involve sample collection from pregnant women.	To obtain approval from the MoHSS of Namibia and submit copy to FHAS-REC secretariat*.
2.	Research activities involve collection of information/data from minor.	To provide consent form to be used with the questionnaire

NB: May attach additional page as required; * Failure to do so will invalidate research outcome

Full Name (reviewer): ...Ms Berta van der Colf...Signature: ..... Date: 5.7.17

Full Name: ...PROF OR AWOFOLU..... Signature: ..... Date: 2/9/2017
Chair: Ethics Screening Committee

APPENDIX F: APPROVAL BY THE NAMIBIA INSTITUTE OF PATHOLOGY

OFFICE OF THE CHIEF OPERATIONS OFFICER

Enquiries: Mr Boniface Makumbi; Tel.: 061-295 4210

Date: 01 November 2017

Mr Erastus L Haimbodi
Namibia University of Science and Technology (NUST)
Windhoek
Namibia

Dear Mr Haimbodi

RE: **Prevalenceand Molecular Characterisation of group B Streptococcus in Pregnant Women from Hospitals in Oshikoto and Ohangwena Regions of Namibia**

1. The above mentioned research proposal was referred to the Research/Ethics Committee of the Namibia Institute of Pathology Limited for review.
2. It is a pleasure to inform you that approval was granted for you to proceed with the research on condition that the following be complied with:

2.1 Observe and adhere to all ethical considerations and confidentiality to protect patient information.
2.2 Adhere to all terms and conditions as stipulated by the Ministry of Health and Social services
2.3 Final report to be shared with the Namibia Institute of Pathology Limited.

Yours Sincerely

pp .....

Harold T Kaura
Chief Operations Officer